Method of Detecting Microorganisms in Products

The invention comprises methods of detecting microbial contaminations in non-sterile products, preferably according to GMP guidelines. Furthermore, the invention comprises a test kit for detecting microbial contaminations and the use of primer sequences and probe sequences to determine microorganisms in products, particularly in drugs and cosmetics, including their starting materials and intermediates.

The method is used in the quantitative identification of microorganisms by detecting specifically amplified DNA sequences and is to replace related methods in European Pharmacopoeia, section 2.6.12-13, 1997 (EP), and in other national monographs such as USP.

The production of drugs and cosmetics according to GMP guidelines involves chemical, physical and biological tests to ensure quality. In the case of cosmetics, the manufacturer has to take care that the final products would not be the source of health hazards (EC Cosmetics Regulation 76, 768 EEC (KOSVO), 6), Amended Regulation EC KOSVO 93/35/EEC, 1993, and requirements of national law in Germany (LMBG, section 24).

In the case of drugs, the microbiological requirements as to purity are much more precise, covering the KOSVO requirements (EP, section 2.6.12-13,1997).

The requirements comprise two groups:

(i) counting the total of viable aerobic bacteria and fungi (total germ number group), and

(ii) detecting the absence of particular microorganisms: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Streptococcus faecalis*, salmonella, and enterobacteriaceae (indicator germ group).

State of the art

Germ count using nutrient media

As to the methods of counting the total of viable aerobic bacteria (total germ number group), the EP describes conventional microbiological techniques involving the growth of microorganisms to be detected in particular nutrient media or on agar plates. Numerous appropriate ready-to-use products or starting materials thereof are commercially available.

The use of the methods described in the EP for determining aerobic germs (total germ number group) has the following drawbacks:

- The efficiency is low because a long time is required to obtain the result (3-5 days).
- The results are imprecise. The acceptable limits may vary by a factor of 5; EP, section 2.6.12.
- The test methods are poor and automatizable to only a low degree.
- Due to the properties of the nutrient media, it is only possible to detect well-growing microorganisms rather than all of the aerobic microorganisms as demanded.
- The storage expenses for media and incubators are high.
- With drugs having bacteriostatic properties, the use of the EP methods partially yields non-utilizable results due to low recovery of test microorganisms added.
- There is extensive plastic waste.
- The cost of energy for preparing media and autoclaving the waste produced is high.

- Fertility testing of all lots of media is highly expensive, particularly due to the short shelf-life of ready-to-use media.

  Alternative commercial methods of determining the total germ number are apparatus operated using laser scan, such as CHEMSCAN (Chemunex):
- This method is inappropriate in detecting microorganisms which, as is the case with the Sarcina genus of bacteria, do not form individual colonies.
- In addition, this method is not suited for solid and greasy products to be tested.

Detection of specific microorganisms via differing culture properties and specific metabolites

As to the methods of determining specific germs (indicator germ group), the EP describes microbiological techniques which, for coarse differentiation, involve the growth of respective microorganisms in specific selective nutrient media or on agar plates. Subsequently, specific metabolic reactions of the respective microorganisms are used for fine differentiation. Appropriate detection systems, e.g. APILAB or VITEK, are widely used.

The use of the methods described in the EP for determining specific germs (indicator germ group) involves the same drawbacks as the use of the methods demanded by the EP for determining the aerobic germs (see above). It is an additional disadvantage that the selectivity of the detecting methods is restricted to differences in metabolism, thus allowing not more than inadequate differentiation.

Detection of specific microorganisms by determining the ATP content following preliminary cultivation

Alternative methods on the market are: microbiological quick tests based on vital detection by ATP determination (e.g. Millipore Company) after propagating the microorganisms in nutrient media.
Disadvantage: determination of species is not possible, and the measured results are subject to high fluctuations depending on the vitality condition, being highly dissimilar in different genera of bacteria.

Detection of specific microorganisms after preliminary cultivation, using DNA probes, primers, and PCR

Other alternative commercial methods are various PCR uses which, however, as in Chen et al., 1997, J. Food Microbiol. 35, 239-250, aim for testing foodstuffs and possibly will not comply with the strict GMP requirements as to testing the quality of drugs.

- As a rule, existing PCR uses are prone to contamination by PCR products, are less reproducible and difficult to quantify. Moreover, they are time-consuming because the alternative PCR procedures normally require several hybridization steps to detect the PCR product.
- In addition, these techniques normally can be automatized to only a limited extent and are liable to give trouble, because various reagents normally have to be added at various times during use.

In the method according to the patents US 4,800,159 and US 4,683,195, the nucleic acid to be amplified, which is single-stranded or is made single-stranded, is treated with a molar excess of two oligonucleotide primers under hybridizing conditions and in the presence of an agent in-

ducing polymerization and nucleotides, the primers being selected such that an extension product of the respective primer, which is complementary to the nucleic acid strand, is synthesized for each strand, and that an extension product of a primer, when separated from its complement, can be used as a template to synthesize an extension product of the other primer. Following removal of the extension products from the templates where they have been synthesized, the extension products formed can be used in another reaction with the primers. Owing to the cyclic repetition of these steps, a theoretically exponential propagation of a nucleic acid sequence results, which is located within the outer hybridization positions of the primer.

Quantitative detection of microorganism DNA using a special fluorescence PCR technique

A refined method is the procedure according to US patent 5,210,015 by Gelfand et al., wherein an oligonucleotide probe construction is used, which undergoes hybridization with part of the nucleic acid strand of the template, the oligonucleotide probe being selected so as to fit between the primer pairs (forward and reverse primer) for the amplification of the diagnostic target sequence of the respective microorganism. Probe construction and synthesis are based on the TaqMan technology (Holland et al. and Lee et al., 1993, Nucl. Acids Res., Vol. 21, pp. 3761-3766).

The chemical basis of this new method is the 5' nuclease PCR assay published in 1991 for the first time (Holland et al. 1991, PNAS USA 88, 7276). The essential part of this method is the 5' nuclease activity of Taq polymerase and the use of fluorescence-labelled, sequence-specific gene probes. These gene probes are labelled at their 5' ends with a fluorescein derivative (reporter) and with a rhodamine derivative (quencher) at their 3' ends. As a re-

sult of the spatial proximity of both dyes, the fluorescence radiation of the reporter is absorbed by the quencher dye. During the polymerase chain reaction (PCR), reporter and quencher are spatially separated from each other by the 5' nuclease activity of the Taq polymerase. The fluorescence radiation of the reporter is no longer quenched and can be measured and quantified directly. The more probes cleaved, the higher the fluorescence emission of the reporter molecules. The amount of liberated emission is proportional to the amount of PCR products formed, which in turn is proportional to the number of copies of genes employed in the PCR. The number of organisms present in the analytical sample can be calculated via the number of gene copies. The method is extremely sensitive because gene propagation and thus, signal amplification occurs during the PCR reaction. Various reporter dyes are available on the market and therefore, internal controls and standards can be included in each reaction. Moreover, a sample can be tested for the presence of multiple genes/organisms at the same time. At present, three different reporter dyes are commercially available.

Problem and solution

The central object of the present invention is the development of detection methods for microorganisms which, according to experience, frequently appear as product contaminants. With respect to the group of indicator germs, in particular, these are: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, salmonella types, and with respect to the total germ number group: bacteria and enterobacteriaceae.

It is the object of the present invention to provide reagents, methods and uses of substances rendering the detection of microbial contaminations of non-sterile prod-

ucts, e.g. according to the requirements of the EP, easier, more precise, and more efficient, where less components are intended to be included compared to e.g. the requirements of the EP. Another object is to provide highly sensitive and quantitative detection of microorganisms as demanded.

Said object is accomplished by means of a test kit for detecting microbial contaminations in non-sterile products, particularly according to GMP guidelines, including cosmetics and foodstuffs, which test kit comprises at least one DNA fragment comprising the following SEQ IDs and spacers:

(a) a forward primer (SEQ ID forward primer);
(b) a probe (SEQ ID probe);
(c) a reverse primer (SEQ ID reverse primer);
(d) optionally a spacer between forward primer and probe,
(e) optionally a spacer between probe and reverse primer,
(f) optionally a spacer upstream from the forward primer,
(g) optionally a spacer downstream from the reverse primer,

the SEQ IDs [(SEQ ID forward primer), (SEQ ID probe), and (SEQ ID reverse primer)] also comprising variants wherein one, two or three nucleotides have been substituted, deleted and/or inserted,

the variant essentially having the same function as the sequence of the SEQ IDs [(SEQ ID forward primer), (SEQ ID probe), and (SEQ ID reverse primer)],

with probes, the function of binding to DNA, and with primers, the function of binding to DNA and providing an extendable 3' end for the DNA polymerase,

the spacers comprising 0-40 nucleotides,

the DNA fragment, selected from the group of

(i) for *Staphylococcus aureus*
SEQ ID No. 6 as forward primer
SEQ ID No. 7 as probe, and
SEQ ID No. 8 as reverse primer

(ii) for *Pseudomonas aeruginosa*

SEQ ID No. 9 as forward primer
SEQ ID No. 10 as probe, and
SEQ ID No. 11 as reverse primer
(iii) for *Escherichia coli*
SEQ ID No. 12 as forward primer
SEQ ID No. 13 as probe, and
SEQ ID No. 14 as reverse primer
(iv) for *Salmonella ssp.*
SEQ ID No. 15 as forward primer
SEQ ID No. 16 as probe, and
SEQ ID No. 17 as reverse primer
(v) for bacteria
SEQ ID No. 18 as forward primer
SEQ ID No. 19 as probe, and
SEQ ID No. 20 as reverse primer
(vi) for enterobacteriaceae
SEQ ID No. 44 as forward primer
SEQ ID No. 46 as probe, and
SEQ ID No. 45 as reverse primer
(vii) for enterobacteriaceae (16S rRNA)
SEQ ID No. 47 as forward primer
SEQ ID No. 48 as probe, and
SEQ ID No. 49 as reverse primer

or additionally all those sequences which are complementary to the above sequences from SEQ ID No. 6 to 49.

A combination of two, more preferably three, and even more preferably four, and most preferably five, six or seven complete sequences is advantageous.
A kit including PCR reagents is preferred.
More preferred is a kit including PCR reagents and TaqMan.

All the above-mentioned sequences are presented in Example 24. For successful TaqMan PCR, the primer and probe sequences (Example 24) are to meet the following requirements:

- The primers should be between 15 and 30 bases long.
- The probe sequence must be located between primer sequences on the DNA to be amplified.
- Optionally, the probe should be between 18 and 30 bases long.
- The probe should have a GC content of 40-60%.
- The Tm of the probe (melting point) should be 5-10°C above the Tm of the primer.
- There should be no G at the 5' end of the probe.
- More than 3 times the same base consecutively should never occur in the probe sequence.
- No complementariness between probe and primers or within the primers, and no conspicuous secondary structures within the probe and the primers.

Despite these general guidelines for designing primers and probes (Livak et al. 1995, Guidelines for designing Taqman fluorogenic probes for the 5' nuclease assays, Perkin Elmer Research News), the optimum primer and probe combination has to be re-determined by experiment for each TaqMan PCR use. Although the above-mentioned guidelines had been observed, it was not possible to develop an optimal TaqMan PCR system, as has been demonstrated in a number of examples (Example 25). On the other hand, the sequence characteristics of the diagnostic target sequence of the respective organism (e.g. high GC content, highly repetitive sequences or conserved sequence regions) possibly necessitate the selection of primer and probe sequences which do not comply with the above-mentioned designing guidelines. As a consequence of such restrictions of the guidelines, the selection of the diagnostic target sequence from the genome of the microorganism to be detected and the experimental determination of optimum primer and probe sequences is essential in achieving the required specificity and sensitivity of a TaqMan PCR test.

PCR reaction conditions, including TaqMan buffer:

Apart from the primer and probe sequences (a-c), the specificity and sensitivity of a TaqMan PCR test is determined by the following parameters:

(i) Level of denaturation temperature in the initial PCR cycles

(ii) Level of annealing temperature during the amplification phase in the PCR

(iii) Number of PCR cycles

(iv) Use of PCR additives such as glycerol and/or formamide

(v) Use of 7-deaza-2-deoxy-GTP in addition to GTP on genes having high G/C content

(vi) Level of Mg^{2+} ion concentration in the PCR buffer

(vii) Concentration of primer and probe

(viii) Amount of Taq DNA polymerase

(ix) Spacing of the *cis*-oriented primer from probe

All these parameters have been contemplated experimentally in the development of the TaqMan PCR tests presented herein (data not shown).

Description of nucleic acids used as diagnostic target sequences

In particular, the nucleic acids used in the amplification procedure and detection procedure of the above-mentioned target organisms are understood to be genomic nucleic acids. Among other things, genomic nucleic acid sequences also include the genes or gene fragments characteristic for a specific species, genus, family, or subclass of microorganisms. The nucleic acid sequences can be used in a PCR test as diagnostic target sequences for specific detection of such a species, genus, family, or subclass.

The following target sequences have been selected to detect the above-mentioned target organisms:

Organism(s)		Designation of gene
(i)	*Staphylococcus aureus*	*cap8*
(ii)	*Pseudomonas aeruginosa*	*algQ*
(iii)	*Escherichia coli*	*murA*
(iv)	*Salmonella ssp.*	*invA*
(v)	Bacteria	16S rRNA

The genes from which the diagnostic target sequences have been selected will be described in detail in the Examples.

Definitions:

Primer definition (including variations thereof):

A primer is understood to be a molecule having a number of nucleotides on a polymer basic skeleton. The sequence of the nucleobases is selected in a way so as to have more than 80% complementariness to successive bases of the nucleotide sequence to be amplified. Each of these molecules has at least one extendable end. In particular, extension is understood to be an enzyme-catalyzed coupling of base units, using mononucleoside triphosphate units or oligonucleotides. A DNA polymerase is preferably used as enzyme. The nucleic acid containing nucleotide sequences to be amplified is used as a template for the specific incorporation of bases. The sequence of the template determines the sequence of bases coupled to the primer. Molecules having 15-30 bases are used as primers. In the event of a DNA polymerase, the 3' end preferably serves as extendable end. Those primers are particularly preferred which are completely homologous to a partial sequence of the target nucleotide sequences SEQ ID No. 1-5 (Example 24).

Probe definition (including variations):

A probe is understood to be a molecule which - just like the primers - has a number of nucleotides on a polymer basic skeleton. Here, a probe construction procedure according to US patent 5,210,015 is used, which already has been described above. The nucleic acid probes of the pres??ent invention are 18-30 nucleobases in length. Specific sequences are obtained by selecting one sequence at least 18 bases in length from the respective templates (SEQ ID No. 1-5, Example 24). According to the invention, probes having at least 90% homology to a part of the respective templates (SEQ ID No. 1-5) are therefore preferred. Probes having strict homology are particularly preferred.

Definition of homology:

The invention is directed to nucleotide sequences being at least 80%, preferably 90%, more preferably 95% complementary to the target nucleotide sequences SEQ ID No. 1 to 5, 46 and 48. The homology (in %) is obtained from the number of identical purine and pyrimidine bases in a given nucleotide sequence.

Definition of hybridization:

Hybridization is present if the following processing steps, and preferably the following conditions have been realized:

The primers and probes according to the invention bind to complementary bases, preferably to complementary nucleotide sequences in the genotype of the target organisms from the total germ number group and to complementary nucleotide sequences in the genotype of the target organisms from the indicator germ group.

Moreover, they preferably will not bind to nucleic acid sequences specific for other microorganisms.

Definition of drugs:

These substances are active substances, raw materials, adjuvants, and formulations described in the monographs of the EP and intended for use in human medicine and veterinary medicine.

Definition of cosmetics:

These substances have not been described in the monographs of the Pharmacopoeia, but are subject to the KOSVO and LMBG directives and comprise raw materials, adjuvants, and formulations intended for use in humans and animals.

Definition of microorganism:

Predominantly, this term comprises organisms which may cause diseases in the human and animal body and are visible only by microscopic means. As a rule, they are unicellular, appearing in loose associations of alike cells, and are referred to as protists due to their simple cellular organization. Their morphologic and cultural-biochemical features, as well as their chemical composition, antigenic properties and genetic features are well-documented in the literature, e.g. in: Mikrobiologische Diagnostik, Burkhardt, 1992.

Definition of PCR reagents:

PCR reagents are substances required for a PCR reaction with maximum sensitivity and specificity, particularly DNA polymerase, Mg^{2+} ions as in $MgCl_2$, potassium salts such as KCl, additives such as glycerol or DMSO or formamide, primers and probes, deoxynucleotides, buffer substances such as Tris base, as well as optional additives in the form of passive fluorescent reference compounds, e.g. the fluorescent dye derivative ROX, and e.g. 7-deaza-2-deoxy-GTP as a substitute for dGTP.

Definition of complementariness:

Complementary structures correspond to or supplement each other. Thus, for example, the polynucleotide strands of the natural DNA double helix are complementary, forming two complementary strands due to specific base pairing (A-T and G-C, respectively). As a result, the nucleotide sequence in the other strand is unambiguously determined, though non-identical, but complementary. Similarly, this applies to DNA-RNA hybrids (having A-U instead of A-T pairs). cDNA has a structure complementary to an mRNA. Those complementary structures are preferred wherein (aa) the sequence of the forward primer and the sequence of the probe or (bb) the sequence of the probe and of the reverse primer of a previously mentioned group from (i) to (vii) both are complementary to the defined sequences. Those complementary structures are more preferred wherein the sequence of the forward primer, the sequence of the probe and of the reverse primer of a previously mentioned group from (i) to (vii), i.e, all of the three above, are complementary to the defined sequences.

Methods

The invention is also directed to a method of detecting microorganisms in products, particularly drugs or cosmetics, said method comprising the following steps:

a) use of primers and fluorescence-labelled probes having the appropriate sequences and variations thereof,

(i) for *Staphylococcus aureus*
SEQ ID No. 6 as forward primer
SEQ ID No. 7 as probe, and
SEQ ID No. 8 as reverse primer

(ii) for *Pseudomonas aeruginosa*
SEQ ID No. 9 as forward primer
SEQ ID No. 10 as probe, and
SEQ ID No. 11 as reverse primer

(iii) for *Escherichia coli*
SEQ ID No. 12 as forward primer
SEQ ID No. 13 as probe, and
SEQ ID No. 14 as reverse primer

(iv) for *Salmonella ssp.*
SEQ ID No. 15 as forward primer
SEQ ID No. 16 as probe, and
SEQ ID No. 17 as reverse primer

(v) for bacteria
SEQ ID No. 18 as forward primer
SEQ ID No. 19 as probe, and
SEQ ID No. 20 as reverse primer

(vi) for enterobacteriaceae
SEQ ID No. 44 as forward primer
SEQ ID No. 46 as probe, and
SEQ ID No. 45 as reverse primer

(vii) for enterobacteriaceae (16S rRNA)
SEQ ID No. 47 as forward primer
SEQ ID No. 48 as probe, and
SEQ ID No. 49 as reverse primer

or additionally all those sequences which are complementary to the above sequences from SEQ ID No. 6 to 49;

b) propagating the DNA using PCR, and

c) irradiating with specific wavelengths exciting the fluorescent dye,

d) measuring and quantifying the emission of the excited fluorescent dye.

The invention comprises an inventive method, the preparation of the probes being based on TaqMan detection techniques.

Essence of the invention

The essence of the invention is the combination of specific, selected probe/primer pairs capable of detecting

microorganisms in a satisfactory fashion. Optimizing the probe/primer pairs and the PCR reaction conditions for sensitivity and suitability for GMP-conforming product testing according to EP, 2.6.12-13: Microbial contamination of products not required to comply with the test for sterility (1997), is also essential. A PCR technology according to the patents US 4,800,159 and US 4,683,195 is used and, in particular, the TaqMan technology described in US patent 5,210,015 issued as patent on May 11, 1993, is employed.

The method according to the invention or the test kit according to the invention is a special embodiment of the fluorescence PCR technology (TaqMan) for the above-mentioned target microorganisms.

Advantages:

In many respects, the methods of the invention and the test kits are far superior to the analytical methods prescribed in the EP (no prescribed methods are demanded for cosmetics as yet) and are intended to completely replace the latter, once the method has been validated on the respective product to be tested. The option of using other analytical methods is explicitly admitted in the EP (General Notices), provided they furnish the same results as the prescribed methods.

In particular, the method according to the invention has the following advantages:

(A) A kit and a method of detecting microorganisms from the total germ number group:

By using said kit and method, the analytical determination of all contaminating bacteria whose sequences are described in the NIH data base, USA, as of 11/1997, is possible for the first time without preceding cultivation, where live bacteria incapable of propagating are detected quantita-

tively with high precision and a sensitivity of 1-3 bacteria in the product to be tested. One consequence of such a use is an improved product safety for the consumer because:

- spores and microorganisms difficult to cultivate, which may be the source of health hazards, can be detected,
- microorganisms incapable of propagation, which contain toxins difficult to detect, can also be detected,
- contaminating DNA of bacterial origin, whose absence in biologicals and products from the rDNA technology already has to be demonstrated even today (EP, 1997, and USP 1995) can be detected easily and efficiently in all the products to be tested.

Furthermore, there are no particular safety instructions because none of the components of the kit is subject to a hazardous material regulation.

(B) All claimed kits and methods:

- Such a use has economic advantages for consumer and manufacturer, because the previous methods are more time-consuming by several days and frequently represent the time-determining step in clearance analytics. Fast results as to the microbiological safety of a biologically sensitive product to be tested result in lower costs in development and production, e.g. lower storage cost, or faster response to variable commercial inquiries and on the whole, in a reduction of production cost, resulting in cheaper products.
- Such a use has ecological advantages, because the reduction in analysis time and analysis material (plastics and media) significantly reduces the energy cost which is considerable.

Examples:

The following Examples describe the developed PCR quick tests for detecting the target microorganisms, including all sequence variations and target sequences:

(i)	*Staphylococcus aureus*	(Examples 1-5)
(ii)	*Pseudomonas aeruginosa*	(Examples 6-9)
(iii)	*Escherichia coli*	(Examples 10-13)
(iv)	*Salmonella ssp.*	(Examples 14-17)
(iv)	Bacteria	(Examples 18-23)
(vi)	Target, probe and primer sequences	(Example 24)
(vii)	Sequence variations	(Example 25)
(viii)	Developed sequences of probes and primers having non-satisfactory test specificity/sensitivity	(Example 26)

Example 1
DNA liberation following initial accumulation

An 100 µl aliquot of each microorganism culture each time was lysed to liberate the DNA (Makino et al., Applied Environ. Microbiol. 3745-3747, 1995). The DNA was purified to remove proteins and other PCR inhibitors and then used in PCR amplification experiments.

Example 2
Detection of *Staphylococcus aureus*

The detection of *S. aureus* was effected by species-specific amplification of cap-8 gene sequences according to the invention (SEQ ID No. 1, see Example 24). The cap-8 gene cluster encodes proteins involved in the biosynthesis of the capsule of *S. aureus*. The capsule covers the surface of these bacteria, representing a protective mechanism against the defence mechanisms of the host organisms. The

molecular composition of the capsule is specific for *S. aureus* and, so to speak, represents a molecular fingerprint of this *Staphylococcus* species. The ORF-O (open reading frame O) of the cap-8 gene cluster is conserved in various serotypes of *S. aureus* (Sau and Lee 1996, J. Bacteriol. 178, 2118-2126). The DNA sequences from the ORF-O of the cap-8 gene cluster (SEQ ID No. 1) were selected as diagnostic DNA sequences to synthesize species-specific DNA primers and probes.

As a result of DNA sequence data base comparisons and practical optimization operations using various primer/probe combinations, the following cap-8-specific DNA sequences were determined as optimum primer/probe combination:

1. PCR probe
20 mer 5'-TAMRA-CCT GGT CCA GGA GTA GGC GG 3'-FAM
(probe cap-8 # 15460*, use as reverse complement) [SEQ ID No. 7].
Probes were produced by the PE Applied Biosystems Division, Weiterstadt, Germany. They are single-stranded oligonucleotides modified at their 5' end with a fluorescent derivative (FAM = 6-carboxyfluorescein) and at their 3' end with a rhodamine derivative (TAMRA = 6-carboxytetramethylrhodamine). Synthesis and purification were performed according to the instructions of PE Applied Biosystems.

2. PCR primer
24 mer: 5'-AGA TGC ACG TAC TGC TGA AAT GAG-3'
(primer cap-8 forward # 15297*) [SEQ ID No. 6]

26 mer: 5'-GTT TAG CTG TTG ATC CGT ACT TTA TT-3'
(primer cap-8 reverse # 15485*, use as reverse complement) [SEQ ID No. 8]

* The positions refer to those in the cap-8 DNA sequence published by Sau and Lee (1996, J. Bacteriol. 178, 2118-2126).
Synthesis and purification of the PCR primer oligonucleotides were performed by PE Applied Biosystems according to their protocols.

Example 3
PCR conditions for the detection of *Staphylococcus aureus*

After varying primer and probe concentrations and $MgCl_2$ concentration, the following conditions were found to be optimal:
All components were purchased from PE Applied Biosystems, Weiterstadt, Germany. Preparation of the TaqMan PCR reaction mixtures, performing the PCR reactions and operating the PCR heating stage and fluorescence detector (PE 30 ABD model 7700 or model LS50B) were according to the instructions of the instrument manufacturer (User's Manual, ABI Prism 7700 Sequence Detection System, PE Applied Biosystems 1997, and User's Manual, PE ABI LS50B).

The following components were mixed in a PCR reaction vessel (PE Applied Biosystems, Order No. N8010580):

Component	Volume (µl)	Final concentration (in 50 µl)	Amount
DNA	5.00		1 fg - 100 ng
Bidist.	10.25		
10fold concentrated TaqMan buffer A*	5.00	1 x	
25 mM $MgCl_2$ solution	8.00	4 mM	
DATP	2.00	200 mM	
DCTP	2.00	200 µM	
DGTP	2.00	200 µM	
DUTP	2.00	400 µM	
5' Primer # 15297	5.00		15 pmol
Probe # 15460	3.00		6 pmol
3' Primer # 15485	5.00		15 pmol
Ampli Taq Gold*	0.25		1.25 units
AmpErase UNG*	0.50		0.50 units
Total volume	50.00		

* (from TaqMan PCR Core Reagents, N 8080229, PE Applied Biosystems)

For optimum reproducibility of the results, care must be taken to premix as many components of the PCR mix as possible in a so-called master mix in each PCR cycle. Under standard conditions, only the DNA material to be tested (0 - 15.25 µl) is added separately as component to each PCR reaction vessel.

The PCR reactions are carried out in the PCR heating stage of the ABI Sequence Detector 7700. Functionally equivalent are PCR heating stages having comparable heating and heat transfer properties, such as the PE ABI apparatus model 7200, 9700, 9600, and 2400.

Cycle	**Temperature (°C)**	**Time (min)**	**Repeats**
Hold	50	2:00	1
Hold	95	10:00	1
Cycle	95	0:15	40
Cycle	60	1:00	
Hold	25	5:00	

For detailed illustrations as to the PCR cycle profile, see: User's Manual, ABI Prism 7700 Sequence Detection System, PE Applied Biosystems 1997.

Example 4
Selectivity of the *S. aureus* PCR quick test

4.1 Electrophoretic analysis

To estimate the selectivity of the PCR test, genomic DNA from various organisms was isolated and employed in the PCR test (Fig. 1, Sambrock et al., 1993). The PCR products having formed were analyzed by electrophoresis. The PCR products had a size of 213 base pairs. Control sequencings of the PCR products confirmed that these were *cap8-0* DNA (not shown).

The DNA (10 ng per lane, 2-14) of all *S. aureus* strains (lanes 2-5) employed was detected by the *cap8-0* primers (# 15297 and # 15485). In contrast, the DNA of a closely related *Staphylococcus* species, i.e., *S. epidermidis* (lane 6) and that of other bacterial genera (lanes 7-11) was not detected. Fungus, fish and human DNAs (lanes 12-14) were used as controls, showing no detection signal. NTC (= no template control) is the water control wherein no DNA was used.

4.2 Fluorescence analysis

In addition to the electrophoretic analysis, the selectivity of the diagnostic PCR was determined as a TaqMan fluorescence test, using the above-mentioned primers and fluorescence probe. The results are given as C_t values (threshold cycle).

C_t value: The hydrolysis of the fluorescence probe occurring during the TaqMan PCR results in an increase of the reporter fluorescence radiation from one PCR cycle to the next. The number of cycles where the reporter fluorescence

radiation is higher than the background radiation (NTC) of the system for the first time and increases linearly is referred to as "threshold cycle" (C_t). (Background radiation (NTC) is the reporter fluorescence radiation in PCR control reactions wherein no template DNA was used.) Both the amount of reporter radiation emitted and "threshold cycle" (C_t threshold value number of cycles) are proportional to the amount of PCR products formed and thus, to the amount of gene copies employed (germ number).

The more gene copies employed, the lower the resulting C_t value. In a PCR system with 100% efficiency, the C_t value will decrease by one cycle each time the starting number of gene copies is doubled. In a PCR reaction comprising e.g. 40 cycles wherein no PCR product is formed, the C_t value will be 40 by definition.

10 ng of template DNA is employed in each PCR reaction for the specificity test. The reaction conditions are specified in Example 3.

List of DNA isolated products tested
(10 ng of genomic DNA analyzed each time)

Organism	**Result** (as C_t value)
***Staphylococcus aureus* species**	
S. aureus	
DSM 683 (ATCC 9144)	17
DSM 1104 (ATCC 25923)	17
DSM 6148	17
DSM 346 (ATCC 6538)	17
S. epidermidis	
DSM 1798 (ATCC 12228)	40

Other bacterial genera	
Organism	**Result** (as C_t value)
Pseudomonas aeruginosa	
DSM 1117 (ATCC 27853)	40
DSM 1128 (ATCC 9027)	40
DSM 3227 (ATCC 19429)	40
DSM 50071 (ATCC 10145)	40
Salmonella typhimurium	
DSM 5569 (ATCC 13311)	40
Streptococcus faecalis	
DSM 2981 (ATCC 14506)	40
(reclassified DSM 2570 (ATCC 29212)	40
as *Enterococcus faecalis*)	
DSM 6134	40
Escherichia coli	
DSM 787 (ATCC 11229)	40
DSM 1576 (ATCC 8739)	40
Eukaryotes	
Neurospora crassa	40
Human (Perkin Elmer ABI, 401846)	40
Salmon (Sigma D 9156)	40
Water	40

After about 17 cycles, a linear increase of the FAM fluorescence above the FAM background radiation of the fluorescence probe was detected for the first time when us-

ing *S. aureus* genomic DNA in the fluorescence PCR. When using DNA from *S. epidermidis* in the PCR, which is a species closely related to *S. aureus* within the *Staphylococcus* genus, no significant increase of the FAM reporter fluorescence could be detected.

The results of the PCR analysis using DNA from various bacterial genera, *Staphylococcus* species and *Staphylococcus aureus* strains demonstrates the specificity of the *S. aureus* test that has been developed. It is only *S. aureus* DNA that is detected by the *cap-8* primers and probes.

Example 5
Sensitivity of the *S. aureus* detection method

To determine the sensitivity of the *S. aureus* PCR test, genomic *S. aureus* DNA was prepared and used in PCR experiments.

10 fg of genomic *S. aureus* DNA correspond to 3 genomes (Strauss and Falkow 1997, Science 276, 707-712).

10 fg = 3 gfu
10 pg = 3,000 gfu
10 ng = 3,000,000 gfu

Various amounts of *S. aureus* DNA (from 1 fg to 100 ng) were used in the fluorescence PCR (Fig. 2). The data shown represent mean values from 6 independent experiments. The amount of emitted fluorescence and thus, of PCR products having formed is given as C_t value.

The result shows that the DNA from 3 bacterial cells can be detected by means of fluorescence PCR. The PCR quick test allows linear quantification of the employed *S.*

aureus genomes over 5 log levels, i.e., between 3 and 300,000 gfu (1 ng of DNA).

Example 6
Detection of *Pseudomonas aeruginosa*

The detection of *Pseudomonas aeruginosa* was performed by species-specific amplification of *algQ* gene sequences according to the invention (for sequences, see Example 24). The *algQ* gene encodes elements of a protective mechanism developed by *Pseudomonas aeruginosa* in the course of evolution, which mechanism is specific for this bacterial species.

The production of alginate is a unique virulence property of *Pseudomonas aeruginosa*. Alginate is a polymer of mannuronic and guluronic acid (1,4-glycosidic linkage). This polymer forms a viscous gel on the bacterial surface. The production of this biogel is subject to highly sensitive regulation. The ability of synthesizing alginate is present in all *Pseudomonas aeruginosa* strains and is characteristic for this species of bacteria. Alginate synthesis is an energy-consuming process and therefore subject to regulation. A gene that regulates the alginate synthesis is the *algQ* gene (Konyecsni and Deretic 1990, J. Bacteriol. 172, 2511-2520). It encodes the sensory component of a signal transduction system (Roychoudhury et al. 1993, PNAS USA 90, 965-969). Because the *algQ* gene is involved in the regulation of a specific protection mechanism, it represents a genetic marker having diagnostic potency in the identification of the *Pseudomonas aeruginosa* species.

As a result of DNA sequence data base comparisons and practical optimization operations using various primer/probe combinations, the following *algQ*-specific DNA

sequences were determined as optimum primer/probe combination:

1. PCR probe:

26 mer: 5'-FAM - **CCA ACG CCG AAG AAC TCC AGC ATT TC** – TAMRA
(Probe *algQ* # 911): [SEQ ID No. 10]

The probes were produced by the PE Applied Biosystems Division, Weiterstadt, Germany. They are single-stranded oligonucleotides modified at their 5' end with a fluorescent derivative (FAM = 6-carboxyfluorescein) and at their 3' end with a rhodamine derivative (TAMRA = 6-carboxytetramethylrhodamine). Synthesis and purification were performed according to the instructions of PE Applied Biosystems.

2. PCR primers:

23 mer: 5'-**CTT CGA TGC CCT GAG CGG TAT TC**-3'
(Primer *algQ* forward # 876*) [SEQ ID No. 9]

Reverse primer sequence (# 1147):
23 mer: 5'-**CTG AAG GTC CTG CGG CAA CAG TT**-3'
(Primer *algQ* reverse # 1147*, use as reverse complement) SEQ. ID. NO. 11

* The positions refer to the DNA sequence published in Konyecsni and Deretic 1990, J. Bacteriol. 172, 2511-2520.
Synthesis and purification of the PCR primer oligonucleotides were performed by PE Applied Biosystems according to their protocols.

Example 7
PCR conditions for the detection of *P. aeruginosa*

After varying primer and probe concentrations and $MgCl_2$ concentration, the following conditions were found to be optimal:

Component	Volume (µl)	Final concentration (in 50 µl)	Amount DNA
	5.00		1 fg - 100 ng
Bidist.	7.25		
10 x TaqMan buffer A	5.00	1 x	
25 mM $MgCl_2$ solution	13.00	6.5 mM	
dATP	2.00	200 µM	
dCTP	2.00	200 µM	
dGTP	2.00	200 µM	
dUTP	2.00	400 µM	
5' Primer # 876	1.00		3 pmol
Probe # 911	4.00		8 pmol
3' Primer # 1147	5.00		15 pmol
AmpliTaq Gold	0.25		1.25 units
AmpErase UNG	0.50		0.50 units
DMSO	1.00		
	50.00		

For optimum reproducibility of the results, care must be taken to premix as many components of the PCR mix as possible in a so-called master mix in each PCR cycle. Under standard conditions, only the DNA material to be tested (0 - 15.25 µl) is added separately as component into each PCR reaction vessel.
The PCR reactions are carried out in the PCR heating stage of the ABI Sequence Detector 7700. Functionally equivalent are PCR heating stages having comparable heating and heat transfer properties, such as the PE ABI apparatus model 7200, 9700, 9600, and 2400.

The PCR cycle profile for the *Pseudomonas aeruginosa* PCR is as follows:

Cycle	Temperature (°C)	Time (min)	Repeats
Hold	50	2:00	1
Hold	95	10:00	1
Cycle	97	0:30	4
	60	1:00	
Cycle	94	0:30	41
	60	1:00	
Hold	25	5:00	

For details as to the PCR conditions, cf. Example 3.

Example 8

Selectivity of the *Pseudomonas aeruginosa* PCR quick test

To estimate the selectivity of the PCR test, genomic DNA from various organisms was isolated and employed in the fluorescence PCR test. The amount of PCR products having formed is given as C_t value (threshold cycle, for C_t value see definition in Example 4).

List of DNA isolated products tested
(10 ng of genomic DNA analyzed each time)

Organism		**Result** (as C_t value)
***Pseudomonas* species**		
P. aeruginosa	DSM 1117 (ATCC 27853)	19
	DSM 1128 (ATCC 9027)	19
	DSM 3227 (ATCC 19429)	19
	DSM 50071 (ATCC 10145)	19
P. putida	DSM 50026	45
P. fluoreszenz	ATCC 948	45
Other bacterial species		
Staphylococcus aureus	DSM 683	45
	DSM 1104	45
	DSM 6148	45
	DSM 6538P	45
Streptococcus faecalis	DSM 2981	45
	DSM 6134	45
	ATCC 29212	45
Salmonella typhimurium	ATCC 13311	45
Escherichia coli	DSM 301	45
	DSM 787	45
	DSM 1103	45
	ATCC 8739	45
Eukaryotes		
Neurospora crassa		45
Arabidopsis thaliana		45
Salmon (Sigma D9156)		45
Human (Perkin Elmer ABI, 401846)		45
Water		45

Only *Pseudomonas aeruginosa* strains gave a positive result in the PCR quick test. After 19 PCR cycles (C_t = 19), a linear increase in fluorescence was measurable for the first time when using 10 ng of *P. aeruginosa* DNA. The PCR test was highly specific. Even the closely related species *P. putida* and *P. fluoreszenz* gave no fluorescence signal in the PCR quick test.

As a positive control, the same bacterial DNAs analyzed in the *algQ* PCR test were examined using the universal 16S rRNA PCR system (see Example 19). All bacterial DNAs gave a positive signal with the 16S rRNA system. Thus, all DNAs allowed amplification by 16S rRNA PCR, but only the *P. aeruginosa* DNA allowed *algQ* PCR amplification. The *algQ* system is *Pseudomonas aeruginosa*-specific.

In addition, the PCR products having formed were analyzed by electrophoresis (cf., Example 3). The PCR products had a size of 294 base pairs (not shown). Control sequencings of the PCR products confirmed that this was *algQ* DNA (not shown).

Example 9
Sensitivity and linearity of the *P. aeruginosa* PCR quick test

To determine the sensitivity of the *P. aeruginosa* PCR test, genomic *P. aeruginosa* DNA was prepared and used in PCR experiments (Fig. 3). Various amounts of *P. aeruginosa* genome copies were used in the fluorescence PCR (Fig. 3). The data shown represent mean values and standard deviations from 4 independent experiments. The amount of emitted fluorescence and thus, of PCR products having formed is given as C_t value. The PCR reaction was performed over 45 cycles. The C_t value of the water control (NTC = no template control) was 45.

The result shows that the DNA from 3 bacterial cells can be detected by means of fluorescence PCR. The PCR quick test allows linear quantification of the employed *P. aeruginosa* genomes over 4 log levels, i.e., between 3 and 30,000 gfu.

Example 10
Detection of *Escherichia coli*

The detection of *E. coli* was performed by species-specific amplification of *murA* gene sequences.

Specific regions of the *murA* gene were used as diagnostic target for the development of a PCR quick test to detect *Escherichia coli*. Why select this gene as a diagnostic target? The *murA* gene encodes the enzyme UDP-N-acetylglucosamineenolpyruvyl transferase, an important structural gene of *E. coli* (Marquardt et al. 1992, J. Bacteriol. 174, 5748-5752). This enzyme catalyzes the first step of the peptidoglycan synthesis, which is murein in the case of *E. coli* and represents an essential component of the bacterial cell wall. The composition of the cell wall is to be regarded as a characteristic feature of bacterial species. The *murA* nucleotide sequence of *E. coli* was compared to that of the closely related enterobacteriaceae species *Enterobacter cloacae*. Owing to the sequence dissimilarities identified, the *murA* gene was selected as a genetic marker having diagnostic potency to identify the enterobacteriaceae species *Enterobacter cloacae*.

As a result of DNA sequence data base comparisons and practical optimization operations using various primer/probe combinations, the following *murA*-specific DNA sequences were determined as optimum primer/probe combination:

Forward primer sequence (# 767*):
5' **GTT CTG TGC ATA TTG ATG CCC GCG** 3' [SEQ ID No. 12]

Probe (# 802):
5'-FAM - **TCT GCG CAC CTT ACG ATC TGG TT** - TAMRA 3' [SEQ ID No. 13]

Reverse primer sequence (# 884):

5' **GCA AGT TTC ACT ACC TGG CGG TTG** 3'

(use as reverse complement) [SEQ ID No. 14]

* The positions refer to the DNA sequence (gene bank: M92358) published in Marquardt et al. 1992, J. Bacteriol. 174, 5748-5752.
The probes were produced by the PE Applied Biosystems Division, Weiterstadt, Germany. They are single-stranded oligonucleotides modified at their 5' end with a fluorescent derivative (FAM = 6-carboxyfluorescein) and at their 3' end with a rhodamine derivative (TAMRA = 6-carboxytetramethylrhodamine). Synthesis and purification were performed according to the instructions of PE Applied Biosystems.

Example 11

PCR conditions for the detection of *Escherichia coli*

After varying primer and probe concentrations, $MgCl_2$ and glycerol concentrations, as well as nucleotide composition, the following conditions were found to be optimal:

Component	Volume (µl)	Final concentration (in 50 µl)	Amount
DNA	5.00		1 fg - 100 ng
Bidist.	8.75		
10 x TaqMan buffer A	5.00	1 x	
25 mM $MgCl_2$ solution	7.00	3.5 mM	
dATP	2.00	200 µM	
dCTP	2.00	200 µM	
7-deaza-dGTP	2.00	200 µM	
dUTP	2.00	400 µM	
Glycerol 40%	2.50	2%	
5' Primer # 767	5.00		15 pmol
Probe # 802	3.00		6 pmol
3' Primer # 884	5.00		15 pmol
AmpliTaq Gold	0.25		1.25 units
AmpErase UNG	0.50		0.50 units

50.00

The PCR cycle profile for the *Escherichia coli* PCR:

Cycle	Temperature (C°)	Time (min)	Repeats
Hold	50	2:00	1
Hold	95	10:00	1
Cycle	95	0:15	40
	60	1:00	
Hold	25	5:00	

For details see Example 3.

Example 12

Selectivity of the *Escherichia coli* PCR quick test

To estimate the selectivity of the PCR test, genomic DNA from various organisms was isolated and employed in the fluorescence PCR test. The amount of PCR products having formed is given as C_t value (threshold cycle; Table).

List of DNA isolated products tested
(10 ng of genomic DNA analyzed each time)

Organism		Result (as C_t value)
Escherichia coli strains		
Escherichia coli		
DSM 301		16
DSM 787		16
DSM 1103		16
ATCC 8739		16
Other enterobacteriaceae		
Acetobacter pasteurianus	DSM 3509	40
Acinetobacter calcoaceticus	DSM 6962	40
Aeromonas enteropelogenes	DSM 6394	40
Alcaligenes faecalis	DSM 30030	40
Budvicia aquatica	DSM 5075	40
Buttiauxella agrestis	DSM 4586	40

Cedecea davisae	DSM 4568	40
Chromobacterium violaceum	DSM 30191	40
Enterobacter cloacae	DSM 30054	40
Edwardsiella tarda	DSM 30052	40
Ewingella americana	DSM 4580	40
Erwinia amylovora	DSM 30165	40
Hafnia alvei	DSM 30163	40
Haemophilus influenzae	DSM 4690	40
Halomonas elongata	DSM 2581	40
Helicobacter pylori	DSM 4867	40
Kluyvera ascorbata	DSM 4611	40
Leclercia adecarboxylata	DSM 5077	40
Legionella pneumophilia	DSM 7515	40
Leminorella grimontli	DSM 5078	40
Levinea malonatica	DSM 4596	40
Listeria monocytogenes	DSM 20600	40
Moellerella wisconsensis	DSM 5076	40
Morganella morganii sp.	DSM 30164	40
Pantoea agglomerans	DSM 3493	40
Photorhabdus luminescens	DSM 3368	40
Plesiomonas shigelloides	DSM 8224	40
Pragia fontium	DSM 5563	40
Providencia stuarti	DSM 4539	40
Proteus mirabilis	DSM 788	40
Rhanella aquatilis	DSM 4594	40
Serratia marcescens	DSM 30121	40
Tatumella ptyseos	DSM 5000	40
Vibrio proteolyticus	DSM 30189	40
Xenorhabdus nematophilus	DSM 3370	40
Yersinia enterocolitica	DSM 4780	40
Other bacterial species		
Pseudomonas aeruginosa	DSM 1128 (ATCC 9027)	40
Bacillus subtilis		40
Salmonella typhimurium	ATCC 13311	40
Pseudomonas mirabelis	DSM 788	40
Staphylococcus aureus	DSM 6538P	40
Streptococcus faecalis	DSM 2981	40
Klebsiella pneumonia	ATCC 10031	40
Citrobacter freundii	DSM 30040	40
Eukaryotes		
Neurospora crassa		40
Arabidopsis thaliana		40
Salmon (Sigma D9156)		40
Human (Perkin Elmer ABD, 401846)		40
Water		40

Only *Escherichia coli* strains gave a positive result in the PCR quick test. After 16 PCR cycles (C_t = 16), a linear increase in fluorescence was measurable for the first time when using 10 ng of *Escherichia coli* DNA. The PCR test was highly specific. Even a closely related enterobacteriaceae species, *Enterobacter cloacae*, gave no fluorescence signal in the PCR quick test (Table).

As a positive control, the same bacterial DNAs analyzed in the *murA* PCR test (Table) were examined using the universal 16S rRNA PCR system (see Example 19). All bacterial DNAs gave a positive signal with the 16S rRNA system, i.e., all DNAs allowed amplification by 16S rRNA PCR, but only the *Escherichia coli* DNA allowed *murA* PCR amplification. The *murA* system is specific for *Escherichia coli*.

In addition, the PCR products having formed were analyzed by electrophoresis (cf., report on *Staphylococcus aureus*). The PCR products had a size of 142 base pairs (not shown). Control sequencings of the PCR products confirmed that this was *murA* DNA (not shown).

Example 13
Sensitivity of the *E. coli* test

To determine the sensitivity of the *Escherichia coli* PCR test, genomic *Escherichia coli* DNA was prepared and used in PCR experiments (Fig. 4).
Varying amounts of *Escherichia coli* genome copies were used in the fluorescence PCR (Fig. 4). The data shown represent mean values and standard deviations from 4 independent experiments. The amount of emitted fluorescence and thus, of PCR products having formed is given as C_t value. The PCR reaction was performed over 40 cycles. The C_t value of the water control (NTC = no template control) was 40.

The result shows that the DNA from 3 bacterial cells can be detected by means of fluorescence PCR. The PCR quick test allows linear quantification of the employed *Escherichia coli* genomes over 6 log levels, i.e., between 3 and 3,000,000 gfu.

Example 14
Detection of *Salmonella ssp.* (subspecies)

The detection of *Salmonella ssp.* of the species *Salmonella enterica* was performed using the specific amplification of *invA* gene sequences according to the invention.

Specific regions of the *invA* gene were used as diagnostic target for the development of a PCR quick test to detect *Salmonella ssp.* Why select this gene as a diagnostic target? The *invA* gene encodes a salmonella-specific virulence factor. Various investigations on a number of salmonella have demonstrated that these bacterial species bind to epithelial cells. In this process, the actin system of the host cells is affected by the bacteria. As a response, the host cells enclose the bacterial cells. After complete enclosure, the bacteria exist in vesicles in the cytoplasm of the host cells. So-called *inv* genes (*invA-H*) of *Salmonella* are involved in this invasion process. Mutants in the *invA* gene still bind to the host cells but are no longer incorporated by same. The *inv* gene sequence is highly conserved in *Salmonella* subspecies (Salyers and Whitt 1994, Salmonella Infection, in: Bacterial Pathogenesis, ASM Press, Washington D.C., p. 233). The *invA* gene of *Salmonella* has been isolated and its nucleotide sequence elucidated (Galan and Curtis 1989, PNAS USA 86, 6383-7; Galan and Curtis 1991, Infection and Immunity 59, 2901-2908, and see: Yards et al. 1992, Mol. Cell. Probes 6, 271-279). The *invA* gene is involved in the expression of a specific virulence mechanism of salmonella and therefore is a genetic marker

having diagnostic potency in identifying *Salmonella ssp.* (Rahn et al. 1992, Mol. Cell. Probes. 6, 271-279).

As a result of DNA sequence data base comparisons and practical optimization operations using various primer/probe combinations, the following *invA*-specific DNA sequences were determined as optimum primer/probe combination:

Forward primer sequence (# 269*):
5' **GTG AAA TTA TCG CCA CGT TCG GGC** 3' [SEQ ID No. 15]

Probe (# 333):
5'-FAM - **CTT CTC TAT TGT CAC CGT GGT CCA** - TAMRA 3' [SEQ ID No. 16]

Reverse primer sequence (# 542):
5' **GGT TCC TTT GAC GGT GCG ATG AAG** 3' [SEQ ID No. 17]
(use as reverse complement)

* The positions refer to the DNA sequence (gene bank: U43237) published in Boyd et al. 1996, Appl. Environ. Microbiol. 62, 804-808.

The probes were produced by the PE Applied Biosystems Division, Weiterstadt, Germany. They are single-stranded oligonucleotides modified at their 5' end with a fluorescent derivative (FAM = 6-carboxyfluorescein) and at their 3' end with a rhodamine derivative (TAMRA = 6-carboxytetramethylrhodamine). Synthesis and purification were performed according to the instructions of PE Applied Biosystems.

Example 15

PCR conditions for the detection of salmonella

After varying primer and probe concentrations and $MgCl_2$ concentration, the following conditions were found to be optimal:

Component	Volume (µl)	Final concentration (in 50 µl)	Amount
DNA	5.00		1 fg - 100 ng
Bidist.	11.25		
10 x TaqMan buffer A	5.00	1 x	
25 mM $MgCl_2$ solution	7.00	3.5 mM	
dATP	2.00	200 µM	
dCTP	2.00	200 µM	
dGTP	2.00	200 µM	
dUTP	2.00	400 µM	
5' Primer # 269	5.00		15 pmol
Probe # 333	3.00		6 pmol
3' Primer # 542	5.00		15 pmol
AmpliTaq Gold	0.25		1.25 units
AmpErase UNG	0.50		0.50 units
	50.00		

The PCR cycle profile for the *Salmonella ssp.* PCR:

Cycle	Temperature (°C)	Time (min)	Repeats
Hold	50	2:00	1
Hold	95	10:00	1
Cycle	95	0:15	40
	60	1:00	
Hold	25	5:00	

For details see Example 3.

Example 16

Selectivity of the *Salmonella ssp.* PCR quick test

To estimate the selectivity of the PCR test, genomic DNA from various organisms was isolated and employed in the fluorescence PCR test. The amount of PCR products having formed is given as C_t value (threshold cycle, for C_t definition, see Example 4).

List of DNA isolated products tested
(10 ng of genomic DNA analyzed each time)

Organism		Result (as C_t value)
Salmonella enterica		
Subspecies		
Salmonella typhimurium	ATCC 13311	15
Salmonella typhi		15
Salmonella agona		15
Salmonella borismorbificans		15
Salmonella anatum		15
Salmonella brandenburg		15
Salmonella derby		15
Salmonella montevideo		15
Salmonella newport		15
Salmonella parathyphi B		15
Salmonella pullorum		15
Salmonella dublin		15
Salmonella enteritidis		15
Salmonella hadar		15
Salmonella infantis		15
Other bacterial species		
Pseudomonas aeruginosa	DSM 1117 (ATCC 27853)	40
	DSM 1128 (ATCC 9027)	40
	DSM 3227 (ATCC 19429)	40
	DSM 50071 (ATCC 10145)	40
Pseudomonas mirabelis	DSM 788	40
Staphylococcus aureus	DSM 683	40
	DSM 1104	40
	DSM 6148	40
	DSM 6538P	40
Streptococcus faecalis	DSM 2981	40
	DSM 6134	40

	ATCC 29212	40
Escherichia coli	DSM 301	40
	DSM 787	40
	DSM 1103	40
	ATCC 8739	40
Enterobacter cloacae	DSM 30054	40
Klebsiella pneumonia	ATCC 10031	40
Citrobacter freundii	DSM 30040	40
Eukaryotes		
Neurospora crassa		40
Arabidopsis thaliana		40
Salmon (Sigma D9156)		40
Human (Perkin Elmer ABD, 401846)		40
Water		40

Only salmonella gave a positive result in the PCR quick test. After 15 PCR cycles (C_t = 15), a linear increase in fluorescence was measurable for the first time when using 10 ng of *Salmonella ssp.* DNA. The PCR test was highly specific. Even the closely related *Escherichia coli* strains gave no fluorescence signal in the PCR quick test.

As a positive control, the same bacterial DNAs analyzed in the *invA* PCR test were examined using the universal 16S rRNA PCR system. All bacterial DNAs gave a positive signal with the 16S rRNA system. Thus, all DNAs allowed amplification by 16S rRNA PCR, but only the *Salmonella* DNA allowed *invA* PCR amplification. The *invA* system is specific for *Salmonella*.

In addition, the PCR products having formed were analyzed by electrophoresis. The PCR products had a size of 287 base pairs (not shown). Control sequencings of the PCR products confirmed that this was *invA* DNA (not shown).

Example 17
Sensitivity of the PCR quick test

To determine the sensitivity of the *Salmonella ssp.* PCR test, genomic *Salmonella typhimurium* DNA was prepared and used in PCR experiments (Fig. 5). Various amounts of *Salmonella typhimurium* genome copies were used in the fluorescence PCR (Fig. 5). The data shown represent mean values and standard deviations from 4 independent experiments. The amount of emitted fluorescence and thus, of PCR products having formed is given as C_t value. The PCR reaction was performed over 40 cycles. The C_t value of the water control (NTC = no template control) was 40.

The result shows that the DNA from 3 bacterial cells can be detected by means of fluorescence PCR. The PCR quick test allows linear quantification of the employed *Salmonella typhimurium* genomes over 6 log levels, i.e., between 3 and 3,000,000 gfu.

Example 18
DNA liberation without previous accumulation in nutrient media

DNA from various test microorganisms was extracted according to Boom et al., 1990, purified to remove proteins and other PCR inhibitors (Quiagen Column Kit, 1995), and used in PCR amplification experiments.

Example 19
Detection of bacteria, universal

The detection of bacteria was performed by the specific amplification of conserved 16S rRNA gene sequences (SEQ ID No. 5, see Example 24) according to the invention. Certain 16S rRNA-specific DNA sequences have become con-

served in the course of evolution; therefore, they are present in the genome of all bacteria and can be used as primers and probes in the universal detection of bacteria (Relman 1993, Weisburg et al. 1991, J. Bacteriol. 173).

As a result of DNA sequence data base comparisons and practical optimization operations using various primer/probe combinations, the following 16S rRNA-specific DNA sequences were determined as optimum primer/probe combination:

1. PCR probe

23 mer: 5'- FAM - **TTA AGT CCC GCA ACG AGC GCA AC** - TAMRA - 3'
(Probe 16S rRNA # 1090): [SEQ ID No. 19]

Probes were produced by the PE Applied Biosystems Division, Weiterstadt, Germany. They are single-stranded oligonucleotides modified at their 5' end with a fluorescent derivative (FAM = 6-carboxyfluorescein) and at their 3' end with a rhodamine derivative (TAMRA = 6-carboxytetramethylrhodamine). Synthesis and purification were performed according to the instructions of PE Applied Biosystems.

2. PCR primers

19 mer: 5'- **GCA TGG CTG TCG TCA GCT C** - 3'
(Primer 16S rRNA forward # 1053*) [SEQ ID No. 18]

20 mer: 5'- **TGA CGG GCG GTG TGT ACA AG** - 3'
(Primer 16S rRNA reverse # 1386*) [SEQ ID No. 20]

* The positions refer to the DNA sequence of the 16S rRNA gene (E. coli in Weisburg et al. 1991, J. Bacteriol. 173)

Synthesis and purification of the PCR primer oligonucleotides were performed by PE Applied Biosystems according to their protocols.

Example 20

PCR conditions for the universal detection of bacteria

After varying primer and probe concentrations and $MgCl_2$ concentration, temperature and cycle profile of the PCR and spacing of the reporter dye from the quencher dye, the following conditions were found to be optimal:
The following components were mixed in a PCR reaction vessel (PE Applied Biosystems, Order No. N8010580):

Component	Volume (µl)	Final concentration (in 50 µl)	Amount
DNA	1.00		1 fg - 100 ng
Bidist. Water	17.25		
10 x TaqMan buffer A	5.00	1 x	
25 mM $MgCl_2$ solution	11.00	5.5 mM	
dATP	1.00	200 µM	
dCTP	1.00	200 µM	
dGTP	1.00	200 µM	
dUTP	1.00	400 µM	
5' Primer #1053	5.00	400 nM	20 pmol
Probe #1090	1.00	40 nM	2 pmol
3' Primer #1386	5.00	400 nM	20 pmol
AmpliTaq	0.25		1.25 units
AmpErase UNG	0.50		0.50 units
	50.00		

For optimum reproducibility of the results, care must be taken to premix as many components of the PCR mix as possible in a so-called master mix in each PCR cycle. Under standard conditions, only the DNA material to be tested (0 - 15.25 µl) is added separately as component to each PCR reaction vessel.
The PCR cycle profile is as follows:

Cycle	Temperature (°C)	Time (min)	Repeats
Hold	50	2:00	1
Hold	95	10:00	1
Cycle	95	0:15	40
Cycle	60	1:00	
Hold	25	5:00	

This regimen is compatible for PCR apparatus with heating stage, e.g. GeneAMP PCR apparatus 2400 and 9600, and ABI Prism 7700 Sequence Detection System by Perkin Elmer. For details, see Example 3.

After completing the PCR reactions, the samples were transferred to the Fluorimeter LS-50B including an additional unit to detect fluorescence in microtiter plates by Perkin Elmer Company. Measurement and quantification of the fluorescence radiation were performed according to the manufacturer's instructions (PE Applied Biosystems, Weiterstadt, Germany).

Example 21
Selectivity of the universal bacterial PCR quick test

To estimate the selectivity of the PCR test, genomic DNA from various organisms was isolated and employed in the universal PCR test (Fig. 6). The amount of PCR products having formed is given in relative fluorescence units (Fig. 6)

The PCR test that has been developed detects bacteria in a selective fashion.

The varying signal intensities of the bacterial samples reflect the variable amounts of DNA employed.

The PCR products having formed were analyzed by electrophoresis. The PCR products had a size of 330 base pairs (not shown). Control sequencings of these PCR products confirmed that this was 16S rRNA (not shown). The PCR quick test is 16S rRNA-specific.

Example 22
Sensitivity and linearity of the quick test for detecting bacteria

To determine the sensitivity of the PCR test, *Salmonella* DNA was prepared and used in PCR experiments. Various dilutions of the DNA were produced. Each dilution was prepared three times in parallel and used in the PCR test (Fig. 7). The amount of emitted fluorescence is given as so-called RQ value.

The RQ value is the difference between the reporter (R) fluorescence radiation in a PCR reaction wherein template DNA (in this case genomic salmonella DNA) is used (R^+) and the reporter fluorescence radiation in a PCR reaction wherein no DNA is used (R^-). Hence, R^- corresponds to the background radiation. The reporter radiation (R) and quencher radiation (Q) are processed into a ratio. The quencher radiation is not subject to change during the PCR reaction, thus representing an internal standard used for standardization.

The result shows that the DNA from 1-3 *Salmonella* bacteria can be detected by means of fluorescence PCR. The fluorescence radiation generated after 40 PCR cycles is significantly above the background radiation.

The fluorescence PCR test allows linear quantification of the employed *Salmonella* genomes over at least 4 log levels, i.e., between 1-3 and 30,000 gfu (Fig. 7).

Example 23
Product testing using the bacterial quick test

The application of the PCR quick test that has been developed was examined using spiking experiments. 10 ml of WFI (water for injection use, Lot No. 63022) was spiked with 50 gfu of salmonella (5 gfu/ml). DNA was prepared from the various spiked samples (Boom et al. 1990), purified (Qiagen 1995), and analyzed in the PCR quick test (Fig. 8).

It was possible to detect the spiked salmonella in the product to be tested. The detected amount was 90% of the amount of DNA employed (Fig. 8). This value reflects the loss of material occurring during the preparation of DNA from the spiked product. Despite such losses, it was possible to detect 1-3 gfu/ml in the spiked product to be tested. On the other hand, no salmonella germs were detected in the non-spiked test product (Fig. 8). The sterility of the test product was demonstrated using membrane filtration according to EP methods (1997).

Example 24
Target gene, primer and probe sequences for the various organisms/groups

SEQ ID No. 1 Staphylococcus aureus

5' **AGATGCACGT ACTGCTGAAA TGAGTAAGCT AATGGAAAAC ACATATAGAG ACGTGAATAT TGCTTTAGCT AATGAATTAA CAAAAATTTG CAATAACTTA AATATTAATG TATTAGTTGT GATTGAAATG GCAAACAAAC ATCCGCGTGT TAATATCCAT CAACCTGGTC CAGGAGTAGG CGGTCATTGT TTAGCTGTTG ATCCGTACTT TATT** 3'

(primer and probe sequences underlined)

SEQ ID No. 6 5' **AGATGCACGT ACTGCTGAAA TGAG** 3'

SEQ ID No. 7 5'- TAMRA - **CCTGGTCCAG GAGTAGGCGG** - FAM -3'

(use as reverse complement)

SEQ ID No. 8 5' **GTTTAGCTGT TGATCCGTAC TTTATT** 3'
(use as reverse complement)

SEQ ID No. 2 Pseudomonas aeruginosa

5' **CAGGCCTTCG ATGCCCTGAG CGGTATTCAG GCACCGGCGC CCAACGCCGA AGAACTCCAG CATTTCTGCC AATTGCTGCT GGACTATGTA TCTGCCGGAC ACTTCGAGGT CTACGAGCAA CTGACGGCGG AAGGCAAGGC CTTCGGCGAT CAGCGCGGCC TGGAGCTGGC CAAGCAGATC TTCCCCCGGC TGGAAGCCAT CACCGAATCC GCGCTGAACT TCAACGACCG CTGCGACAAC GGCGATTGCC GTGAAGGAGC CTGCCTCATC GCGGAGCTGA AGGTCCTGCG GCAACAGTTG CACGAACGCT** 3'
(primer and probe sequences underlined)

SEQ ID No. 9 5' **CTTCGATGCC CTGAGCGGTA TTC** 3'

SEQ ID No. 10 5' - FAM - **CCAACGCCGA AGAACTCCAG CATTTC** - TAMRA - 3'

SEQ ID No. 11 5' **CTGAAGGTCC TGCGGCAACA GTT** 3'
(use as reverse complement)

SEQ ID No. 3 Escherichia coli

5' **AAAGTAGAAC GTAATGGTTC TGTGCATATT GATGCCCGCG ACGTTAATGT ATTCTGCGCA CCTTACGATC TGGTTAAAAC CATGCGTGCT TCTATCTGGG CGCTGGGGCC GCTGGTAGCG CGCTTTGGTC AGGGGCAAGT TTCACTACCT GGCGGTTGTA CGATCGGTGC GCGTCCGGTT GATCTACACA TTTCTGGCCT CGAACAATTA GGCGCGACCA TC** 3'
(primer and probe sequences underlined)

SEQ ID No. 12 5' **GTTC TGTGCATATT GATGCCCGCG** 3'

SEQ ID No. 13 5' - FAM - **TCTGCGCACC TTACGATCTG GTT** - TAMRA - 3'

SEQ ID No. 14 5' **GCAAGT TTCACTACCT GGCGGTTG** 3
(use as reverse complement)

SEQ ID No. 4 Salmonella ssp.

5' TGATTGAAGC CGATGCCGGT GAAATTATCG CCACGTTCGG GCAATTCGTT ATTGGCGATA GCCTGGCGGT GGGTTTTGTT GTCTTCTCTA TTGTCACCGT GGTCCAGTTT ATCGTTATTA CCAAAGGTTC AGAACGTGTC GCGGAAGTCG CGGCCCGATT TTCTCTGGAT GGTATGCCCG GTAAACAGAT GAGTATTGAT GCCGATTTGA AGGCCGGTAT TATTGATGCG GATGCCGCGC GCGAACGGCG AAGCGTACTG GAAAGGGAAA GCCAGCTTTA CGGTTCCTTT GACGGTGCGA TGAAGTTTAT 3'

(primer and probe sequences underlined)

SEQ ID No. 15 5' **GTGAAATTAT CGCCACGTTC GGGC** 3'

SEQ ID No. 16 5' - FAM - **CTTCTCTATT GTCACCGTGG TCCA** - TAMRA - 3'

SEQ ID No. 17 5' **GGTTCCTTTG ACGGTGCGAT GAAG** 3'

(use as reverse complement)

SEQ ID No. 5 bacteria

5' GCATGGCTGT CGTCAGCTCG TGTTGTGAAA TGTTGGGTTA AGTCCCGCAA CGAGCGCAAC CCTTATCCTT TGTTGCCAGC GGTCCGGCCG GGAACTCAAA GGAGACTGCC AGTGATAAAC TGGAGGAAGG TGGGGATGAC GTCAAGTCAT CATGGCCCTT ACGACCAGGG CTACACACGT GCTACAATGG CGCATACAAA GAGAAGCGAC CTCGCGAGAG CAAGCGGACC TCATAAAGTG CGTCGTAGTC CGGATTGGAG TCTGCAACTC GACTCCATGA AGTCGGAATC GCTAGTAATC GTGGATCAGA ATGCCACGGT GAATACGTTC CCGGGCCTTG TACACACCGC CCGTCA 3'

(primer and probe sequences underlined)

(on the example of *E. coli*, Weisburg et al. 1991, J. Bacteriol. 173, 598)

SEQ ID No. 18 5' **GCATGGCTGT CGTCAGCTC** 3'

SEQ ID No. 19 5' - FAM - **TTAAGTCCCG CAACGAGCGC AAC** - TAMRA - 3'

SEQ ID No. 20 5' **CTTGTACACA CCGCCCGTCA** 3'

(use as reverse complement)

SUB B17

Example 25

Variants of primer and probe sequences

Primer/probe sequence combinations are defined as variants, which detect the target DNA sequences with equal specificity (100%) and comparable sensitivity (>70%), such as the sequences specified in Example 24.

Forward primer **Probe** **Reverse primer**

Staphylococcus aureus **(PCR conditions as in Example 3)**

[SEQ.ID.NO 6]AGATGCACGT ACTGCTGAAA TGAG/[SEQ.ID.NO 7]TAMRA-CCTGGTCCAG GAGTAGGCGG-FAM / [SEQ.ID.NO 8]GTTTAGCTGT TGATCCGTAC TTTATT

[SEQ.ID.NO 6] AGATGCACGT ACTGCTGAAA TGAG /[SEQ.ID.NO 7]TAMRA-CCTGGTCCAG GAGTAGGCGG-FAM / [SEQ.ID.NO 23] CATTGTTTAGCTGT TGATCCGTAC T

[SEQ.ID.NO 24]GCACGT ACTGCTGAAA TGAGTAAG/[SEQ.ID.NO 7]TAMRA-CCTGGTCCAG GAGTAGGCGG-FAM / [SEQ.ID.NO 8]GTTTAGCTGT TGATCCGTAC TTTATT

Pseudomonas aeruginosa **(PCR conditions as in Example 7)**

[SEQ.ID.NO 9]CTTCGATGCC CTGAGCGGTA TTC/[SEQ.ID.NO 10]FAM-CCAACGCCGA AGAACTCCAG CATTTC-TAMRA/[SEQ.ID.NO 11]CTGAAGGTCC TGCGGCAACA GTT

[SEQ.ID.NO 25]CAGGCCTTCG ATGCCCTGA GC /[SEQ.ID.NO 10]FAM-CCAACGCCGA AGAACTCCAG CATTTC-TAMRA/[SEQ.ID.NO 11]CTGAAGGTCC TGCGGCAACA GTT

[SEQ.ID.NO 9]CTTCGATGCC CTGAGCGGTA TTC/[SEQ.ID.NO 10]FAM-CCAACGCCGA AGAACTCCAG CATTTC-TAMRA/[SEQ.ID.NO 26]GCTGAAGGTCC TGCGGCAACA G

Escherichia coli (PCR conditions as in Example 11)

[SEQ.ID.NO 12]GTTCTGTGCA TATTGATGCC CGCG/[SEQ.ID.NO 13]FAM-TCTGCGCACC TTACGATCTG GTT-TAMRA/[SEQ.ID.NO 14]GCAAGTTTCA CTACCTGGCG GTTG

[SEQ.ID.NO 27]TAGAACGTAA TGGTTCTGTGC AT/[SEQ.ID.NO 13]FAM-TCTGCGCACC TTACGATCTG GTT-TAMRA /[SEQ.ID.NO 14]GCAAGTTTCA CTACCTGGCG GTTG

[SEQ.ID.NO 12]GTTCTGTGCA TATTGATGCC CGCG /[SEQ.ID.NO 13]FAM-TCTGCGCACC TTACGATCTG GTT-TAMRA/[SEQ.ID.NO 28]CTGGCCTCGA ACAATTAGGC GCG

[SEQ.ID.NO 27]TAGAACGTAA TGGTTCTGTGC AT/ [SEQ.ID.NO 13]FAM-TCTGCGCACC TTACGATCTG GTT-TAMRA /[SEQ.ID.NO 28]CTGGCCTCGA ACAATTAGGC GCG

Salmonella ssp (PCR conditions as in Example 15)

[SEQ.ID.NO 15]GTGAAATTAT CGCCACGTTC GGGC/[SEQ.ID.NO 16]FAM-CTTCTCTATTGTCACCGTGG TCCA-TAMRA/[SEQ.ID.NO 17]GGTTCCTTTG ACGGTGCGAT GAAG

[SEQ.ID.NO 15]GTGAAATTAT CGCCACGTTC GGGC / [SEQ.ID.NO 21] FAM-TT(T/C)GTTATTGGCGATAGCCTGGC-TAMRA /[SEQ.ID.NO 17] GGTTCCTTTG ACGGTGCGAT GAAG

[SEQ.ID.NO 15]GTGAAATTAT CGCCACGTTC GGGC/[SEQ.ID.NO 22] TAMRA-TTCTCTGGATGGTATGCCCGGTA-FAM /[SEQ.ID.NO 17] GGTTCCTTTG ACGGTGCGAT GAAG

Bacteria (PCR conditions as in Example 20)

SUB B2

[SEQ.ID.NO 18]GCATGGCTGT CGTCAGCTC / [SEQ.ID.NO 19]FAM-TTAAGTCCCG CAACGAGCGC AAC-TAMRA / [SEQ.ID.NO 20]CTTGTACACA CCGCCCGTCA

SUB B3

[SEQ.ID.NO 29]TGCATGGCTGT CGTCAGCTC / [SEQ.ID.NO 19]FAM-TTAAGTCCCG CAACGAGCGC AAC-TAMRA / [SEQ.ID.NO 20]CTTGTACACA CCGCCCGTCA

SUB B4

[SEQ.ID.NO 18]GCATGGCTGT CGTCAGCTC / [SEQ.ID.NO 30]FAM-TTGGGTTAAGTCCCG CAACGAGC-TAMRA / [SEQ.ID.NO 20]CTTGTACACA CCGCCCGTCA

Enterobacteriaceae (PCR conditions as in Example 30)

Variants in primer and probe sequences

[SEQ.ID.NO 44]GCATGGCTGT CGTCAGCTC / [SEQ.ID.NO 46]FAM-TTAAGTCCCG CAACGAGCGC AAC-TAMRA / [SEQ.ID.NO 45]TTTATGAGGT CCGCTTGCTC

[SEQ.ID.NO 50]GTGCTGCATG GCTGTCGTC / [SEQ.ID.NO 46]FAM-TTAAGTCCCG CAACGAGCGC AAC-TAMRA / [SEQ.ID.NO 45]TTTATGAGGT CCGCTTGCTC

[SEQ.ID.NO 44]GCATGGCTGT CGTCAGCTC / [SEQ.ID.NO 51]FAM-AGTCCCGCAA CGAGCGCAAC CC-TAMRA / [SEQ.ID.NO 45]TTTATGAGGT CCGCTTGCTC

Example 26
Failure variants in the primer and probe sequences.

Primer/probe sequence combinations are defined as failure variants, which detect the target DNA sequences with non-satisfactory specificity (<100%) and sensitivity (<70%), such as the sequences specified in Example 24. Cf., Figure including primers and probes.

Forward Primer **Probe** **Reverse primer**

Staphylococcus aureus **(PCR conditions as in Example 3)**

[SEQ.ID.NO 31]ATGCACGTAC TGCTGAAATG AG / [SEQ.ID.NO 32] FAM-AACACATATA GAGACGTGAA TATTGC- TAMRA / [SEQ.ID.NO 33] GTTTAGCTGT TGATCCGTAC TT

[SEQ.ID.NO 6]AGATGCACGT ACTGCTGAAA TGAG /[SEQ.ID.NO 32] FAM-AACACATATA GAGACGTGAA TATTGC-TAMRA/[SEQ.ID.NO 23] CATTGTTTAGCTGT GATCCGTAC T

[SEQ.ID.NO 24]GCACGT ACTGCTGAAA TGAGTAAG/[SEQ.ID.NO 32] FAM-AACACATATA GAGACGTGAA TATTGC-TAMRA/[SEQ.ID.NO 8] GTTTAGCTGT TGATCCGTAC TTTATT

Pseudomonas aeruginosa **(PCR conditions as in Example 7)**

[SEQ.ID.NO 9] CTTCGATGCC CTGAGCGGTA TTC/[SEQ.ID.NO 34] FAM - CAATTGCTGC TGGACTATGT ATCTG- TAMRA /[SEQ.ID.NO 1] CTGAAGGTCC TGCGGCAACA GTT

[SEQ.ID.NO 35] CAACGCCGA AGAACTCCAG CATTTC/[SEQ.ID.NO 34] FAM-CAATTGCTGC TGGACTATGT ATCTG-TAMRA/ [SEQ.ID.NO 11] CTGAAGGTCC TGCGGCAACA GTT

[SEQ.ID.NO 9] CTTCGATGCC CTGAGCGGTA TTC/[SEQ.ID.NO 36] FAM-AACGCCGA AGAACTCCAG CATTTCTGC-TAMRA/ [SEQ.ID.NO 26] GCTGAAGGTCC TGCGGCAACA G

[SEQ.ID.NO 9] CTTCGATGCC CTGAGCGGTA TTC/[SEQ.ID.NO 36] FAM-AACGCCGA AGAACTCCAG CATTTCTGC-TAMRA/ [SEQ.ID.NO 11] CTGAAGGTCC TGCGGCAACA GTT

Escherichia coli (PCR conditions as in Example 11)

[SEQ.ID.NO 12] GTTCTGTGCA TATTGATGCC CGCG / [SEQ.ID.NO 13] FAM-TCTGCGCACC TTACGATCTG GTT-TAMRA / [SEQ.ID.NO 37] CATTTCTGGC CTCGAACAAT TA

[SEQ.ID.NO 27] TAGAACGTAA TGGTTCTGTGC AT/[SEQ.ID.NO 38] FAM-CCGCTGGTAG CGCG(T/C)TTTGG TCA-TAMRA/ [SEQ.ID.NO 14] GCAAGTTTCA CTACCTGGCG GTTG

[SEQ.ID.NO 12] GTTCTGTGCA TATTGATGCC CGCG/[SEQ.ID.NO 38] FAM-CCGCTGGTAG CGCG(T/C)TTTGG TCA-TAMRA/[SEQ.ID.NO 37] CATTTCTGGC CTCGAACAAT TA

[SEQ.ID.NO 39] ATGAAGCTGC TAAGCCAGCT GGG /[SEQ.ID.NO 13] FAM-TCTGCGCACC TTACGATCTG GTT-TAMRA / [SEQ.ID.NO 28] CTGGCCTCGA ACAATTAGGC GCG

[SEQ.ID.NO 39] ATGAAGCTGC TAAGCCAGCT GGG/[SEQ.ID.NO 38] FAM-CCGCTGGTAG CGCG(T/C)TTTGG TCA-TAMRA/[SEQ.ID.NO 28] CTGGCCTCGA ACAATTAGGC GCG

[SEQ.ID.NO 39] ATGAAGCTGC TAAGCCAGCT GGG/[SEQ.ID.NO 38] FAM-CCGCTGGTAG CGCG(T/C)TTTGG TCA-TAMRA/ [SEQ.ID.NO 37] CATTTCTGGC CTCGAACAAT TA

[SEQ.ID.NO 39] ATGAAGCTGC TAAGCCAGCT GGG/[SEQ.ID.NO 38] FAM-CCGCTGGTAG CGCG(T/C)TTTGG TCA-TAMRA/ [SEQ.ID.NO 14] GCAAGTTTCA CTACCTGGCG GTTG

Salmonella ssp. (PCR conditions as in Example 15)

[SEQ.ID.NO 40] TTGAAGCCGA TGCCGGTGAA ATTAT/[SEQ.ID.NO 16] FAM-CTTCTCTATTGTCACCGTGG TCCA-TAMRA/[SEQ.ID.NO 17] GGTTCCTTTG ACGGTGCGAT GAAG

[SEQ.ID.NO 40] TTGAAGCCGA TGCCGGTGAA ATTAT/[SEQ.ID.NO 21] FAM-TT(T/C)GTTATTGGCGATAGCCTGGC-TAMRA/ [SEQ.ID.NO 17] GGTTCCTTTG ACGGTGCGAT GAAG

[SEQ.ID.NO 40] TTGAAGCCGA TGCCGGTGAA ATTAT/[SEQ.ID.NO 22] TAMRA-TTCTCTGGATGGTATGCCCGGTA-FAM /[SEQ.ID.NO 17] GGTTCCTTTG ACGGTGCGAT GAAG

[SEQ.ID.NO 40] TTGAAGCCGA TGCCGGTGAA ATTAT/[SEQ.ID.NO 41] FAM-TTTGTTGTCT TCTCTATTGT CACC-TAMRA/[SEQ.ID.NO 17] GGTTCCTTTG ACGGTGCGAT GAAG

[SEQ.ID.NO 15] GTGAAATTAT CGCCACGTTC GGGC/[SEQ.ID.NO 41] FAM-TTTGTTGTCT TCTCTATTGT CACC-TAMRA/[SEQ.ID.NO 17] GGTTCCTTTG ACGGTGCGAT GAAG

Bacteria (PCR conditions as in Example 20)

[SEQ.ID.NO 18] GCATGGCTGT CGTCAGCTC / [SEQ.ID.NO 19] FAM-TTAAGTCCCG CAACGAGCGC AAC-TAMRA / [SEQ.ID.NO 42] AAGTCGTAAC AAGGTAACCA

[SEQ.ID.NO 29] TGCATGGCTG TCGTCAGCTC / [SEQ.ID.NO 19] FAM - TTAAGTCCCG CAACGAGCGC AAC - TAMRA / [SEQ.ID.NO 42] AAGTCGTAAC AAGGTAACCA

[SEQ.ID.NO 43] GGATTAGATA CCCTGGTAGT C / [SEQ.ID.NO 30] FAM - TTGGGTTAAGTCCCG CAACGAGC - TAMRA / [SEQ.ID.NO 20] CTTGTACACA CCGCCCGTCA

[SEQ.ID.NO 43] GGATTAGATA CCCTGGTAGT C / [SEQ.ID.NO 30] FAM - TTGGGTTAAGTCCCG CAACGAGC - TAMRA / [SEQ.ID.NO 42] AAGTCGTAAC AAGGTAACCA

Enterobacteriaceae (PCR conditions as in Example 30)

[SEQ.ID.NO 44] GCATGGCTGT CGTCAGCTC / [SEQ.ID.NO 46] FAM-TTAAGTCCCG CAACGAGCGC AAC-TAMRA / [SEQ.ID.NO 45] TTTATGAGGT CCGCTTGCTC

[SEQ.ID.NO 44] GCATGGCTGT CGTCAGCTC / [SEQ.ID.NO 52] FAM-ATGTTGGGTT AAGTCCCGCA ACG-TAMRA / [SEQ.ID.NO 45] TTTATGAGGT CCGCTTGCTC

[SEQ.ID.NO 50] GTGCTGCATG GCTGTCGTC / [SEQ.ID.NO 52] FAM-ATGTTGGGTT AAGTCCCGCA ACG-TAMRA / [SEQ.ID.NO 45] TTTATGAGGT CCGCTTGCTC

[SEQ.ID.NO 53] GCTGTCGTCA GCTCGTGTT / [SEQ.ID.NO 46] FAM-TTAAGTCCCG CAACGAGCGC AAC-TAMRA / [SEQ.ID.NO 45] TTTATGAGGT CCGCTTGCTC

[SEQ.ID.NO 53] GCTGTCGTCA GCTCGT GTT / [SEQ.ID.NO 46] FAM-TTAAGTCCCG CAACGAGCGC AAC-TAMRA / [SEQ.ID.NO 54] AACTTTATGA GGTCCGCTTG C

[SEQ.ID.NO 44] GCATGGCTGT CGTCAGCTC / [SEQ.ID.NO 46] FAM-TTAAGTCCCG CAACGAGCGC AAC-TAMRA /[SEQ.ID.NO 54] AACTTTATGA GGTCCGCTTG C

Development of a PCR quick test for detecting enterobacteriaceae

The following Examples describe the quick test that has been developed, including all sequence variations and target sequences.

(I) Quick test for detecting enterobacteriaceae, including specification of target, probe and primer sequences (Examples 27-31)

(II) Failure variations in primer and probe sequences (Example 32)

Example 27
Detection of species from the enterobacteriaceae family

To develop a diagnostic PCR quick test for enterobacteriaceae, a gene had to be found which, on the one hand, would have sufficient conserved regions to enable detection of the numerous species of the enterobacteriaceae family and, on the other hand, would also have to contain sufficient variable regions so as to exclude detection of bacteria not belonging to the enterobacteriaceae. By selecting the bacterial 16S rRNA gene as target, both provisos were met.

The 16S rRNA gene encodes the bacterial ribosomal DNA which, together with the 23S rRNA and 5S rRNA, in combination with the ribosomal proteins, forms the translating apparatus for protein biosynthesis.

As a result of DNA sequence data base comparisons and practical optimization operations using various primer/probe combinations, the following specific DNA sequences were determined as optimum primer/probe combination.
As a result of sequence comparisons and practical optimization operations, the following optimum combination of primers and probe was determined for the detection of enterobacteriaceae:

Forward-Primer (#1053) **5´-GCA TGG CTG TCG TCA GCT C-3´** [SEQ ID No. 44]
Reverse-Primer (#1270) **5´-TTT ATG AGG TCC GCT TGC TC-3´** [SEQ ID No.45]

Probe (#1090) **5´-Fam-TTA AGT CCC GCA ACG AGC GCA AC-Tamra-3´** [SEQ ID No. 46]

The probes were produced by the PE Applied Biosystems Division, Weiterstadt, Germany. They are single-stranded oligonucleotides modified at their 5' end with a fluorescent derivative (FAM = 6-carboxyfluorescein) and at their 3' end with a rhodamine derivative (TAMRA = 6-carboxytetramethylrhodamine). Synthesis and purification

were performed according to the instructions of PE Applied Biosystems.

The numerical designations of the oligonucleotides refer to the positions of the main strand of the sequence for the 16S rRNA of *Escherichia coli* published by Brosius et al. in 1978.

The location of these sequences within the 16S rRNA gene is illustrated in SEQ ID No. 24. The size of the amplicon bordered by the primers 1053 and 1270 is 238 bp.

Target sequence of the 16S rRNA gene SEQ ID No. 47

(Forward primer #1053) 5′-**GCATGGCTGTCGTCAGCTC**-3′ from

5′-**CTTCGGGAACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAA** 1082
GAAGCCCTTGGCACTCTGTCCACGACGTACCGACAGCAGTCGAGCACAACACTTT

Sequence Identifier Number 48: (Probe #1090)

5′-FAM-TTAAGTCCCGCAACGAGCGCAAC-TAMRA-3′ from

TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCC 1137
ACAACCCAATTCAGGGCGTTGCTCGCGTTGGGAATAGGAAACAACGGTCGCCAGG
GGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGAC 1192
CCGGCCCTTGAGTTTCCTCTGACGGTCACTATTTGACCTCCTTCCACCCCTACTG
GTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCAT 1247
CAGTTCAGTAGTACCGGGAATGCTGGTCCCGATGTGTGCACGATGTTACCGCGTA
ACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTC 1302
TGTTTCTCTTCGCTGGAGCGCTCTCGTTCGCCTGGAGTATTTCACGCAGCATCAG

Sequence Identifier Number 49: 3′-**TCGTTCGCCTGGAGTATTT**-5′

(Reverse primer #1270)

Location of primers and probe for the specific detection of enterobacteriaceae:

A section of the sequence encoding the 16S rRNA is shown. The digits at the right margin of the sequence indicate the position of each of the last nucleotides in a line. The positions refer to the sequence published by Brosius et al. (1978). Primers and probe are given according to their position in the 16S rRNA gene. FAM: fluorescein derivative as reporter, TAMRA: tetramethylrhodamine derivative as quencher.

Example 28

PCR conditions for the detection of enterobacteriaceae

Composition and components of the TaqMan PCR reaction batch for detecting enterobacteriaceae:
Column 1 lists the single components of the PCR reaction batch. The volumes employed per reaction batch are given in column 2, while column 3 illustrates the final concentration of the single components in the reaction batch. Column 4 indicates the amounts of material of each single component in a 50 µl PCR. UNG: uracil N-glycosylase.

Component	Volume	Final concentration	Amount (in 50µl)
Template (DNA)	5.00 µl	0.1 fg/µl - 20pg/µl	5fg-1ng
Aqua bidist.	11.25 µl	/	/
10x TaqMan buffer A	5.00 µl	1x	/
25 mM $MgCl_2$	7.00 µl	3.5 mM	175 nmol
1,25 mM dATP	2.00 µl	50 µM	2.5 nmol
1,25 mM dCTP	2.00 µl	50 µM	2.5 nmol
1,25 mM dGTP	2.00 µl	50 µM	2.5 nmol
2,50 mM dUTP	2.00 µl	0.1 mM	5.0 nmol
3 µM forward primer #1053	5.00 µl	0.3 µM	15.0 pmol
3 µM reverse primer #1270	5.00 µl	0.3 µM	15.0 pmol
2 µM probe #1090	3.00 µl	0.12 µM	6.0 pmol
5 U/µl AmpliTaq Gold	0.25 µl	25 mU/µl	1.25 U
1 U/µl AmpErase UNG	0.50 µl	10 mU/µl	0.5 U
	Σ 50.0 µl		

The following PCR cycle profile was set up for the detection of enterobacteriaceae:

Step	Time in min	Temperature °C	Repeats
Hold 1	2	50	1
Hold 2	10	95	1
Cycle 1	¼	95	40
	1	60	
Hold 3	2	25	1

PCR profile for the detection of enterobacteriaceae:
Column 1 lists the single components of the PCR reaction batch. The volumes employed per reaction batch are given in column 2, while column 3 illustrates the final concentration of the single components in the reaction batch. Column 4 indicates the amounts of material of each single component in a 50 µl PCR. UNG: uracil N-glycosylase.

Example 29

Selectivity in the detection of enterobacteriaceae:
The Gram-negative family of enterobacteriaceae belongs to the gamma group of the proteobacteria (Balows et al. 1991, Holt 1989). The proteobacteria also include the members of the alpha, beta, delta, and epsilon groups, as well as *Amoebobacter* and some non-classified proteobacteria. Figure 9 shows a rough taxonomic scheme classifying the enterobacteriaceae.

The similarity in the DNA sequences of different species normally increases with increasing degree of relationship. The possibility of an undesirable cross reaction therefore is more likely in closely related species than in less related species. Therefore, the specificity of the developed PCR quick test in the detection of enterobacteriaceae was studied particularly on genomic DNA of close relatives to enterobacteriaceae.

Thirty different enterobacteriaceae species and fourteen bacterial species other than enterobacteriaceae were tested.

All of the tested genera of enterobacteriaceae were detected by the PCR quick test developed herein. In contrast, bacteria strongly related to enterobacteriaceae, particularly including the gamma group members, as well as barely related bacteria, especially the members of *Firmicutes* (Gram-positive bacteria) gave no reaction with the system.

List of tested enterobacteriaceae:

1 ng of genomic DNA of each of the enterobacteriaceae species listed in column 1 was used in the specificity test. The strains employed can be inferred from column 2. Column 3 indicates the result of each test as + (positive reaction) or - (negative reaction) in the PCR quick test for enterobacteriaceae.

Species of family enterobacteriaceae	**Strains**	**Result (+/-)**
Budvicia aquatica	DSM 5075	+
Buttiauxella agrestris	DSM 4586	+
Cedecea davisae	DSM 4568	+
Citrobacter freundii	DSM 30040	+
Edwardsiella tarda	DSM 30052	+
Enterobacter cloacae	DSM 30054	+
Erwinia amylovora	DSM 30165	+
Escherichia coli	ATCC 8739, DSM 301, DSM 787	+
Ewingella americana	DSM 4580	+
Hafnia alvei	DSM 30163	+
Klebsiella pneumoniae	DSM10031	+
Kluyvera ascorbata	DSM 4611	+
Leclercia adecarboxylata	DSM 5077	+
Leminorella grimontli	DSM 5078	+
Levinea malonatica	DSM 4596	+

Moellerella wisconsensis	DSM 5076	+
Morganella morganii	DSM 30164	+
Pantoea agglomerans	DSM 3493	+
Photorhabdus luminescens	DSM 3368	+
Pragia fontium	DSM 5563	+
Proteus mirabilis	DSM 788	+
Providencia stuartii	DSM 4539	+
Rhanella aquatilis	DSM 4594	+
Salmonella typhimurium	ATCC 13311	+
Serratia marcescens	DSM 3370	+
Shigella flexneri	DSM 4782	+
Tatumella ptyseos	DSM 5000	+
Xenorhabdus nematophilius	DSM 3370	+
Yersinia enterocolitica	DSM 4780	+

List of tested bacterial strains not belonging to entero-bacteriaceae:

2 ng of genomic DNA of each of the bacterial species listed in column 1 was used in the specificity test. The membership of species to a particular higher order is shown in column 2. The strains employed can be inferred from column 3. Column 4 indicates the result of each test as + (positive reaction) or - (negative reaction) in the PCR quick test for enterobacteriaceae.

Closely related species of enterobacteriaceae	**Member of**	**Strain**	**Result (+/-)**
Acetobacter pasteurianus	Gamma group	DSM 3509	-
Acinetobacter calcoaceticus	Gamma group	DSM 6962	-
Aeromonas enteropelogenes	Gamma group	DSM 6394	-
Alcaligenes faecalis	Beta group	DSM 30030	-
Chromobacterium violaceum	Beta group	DSM 30191	-
Enterococcus faecalis	Firmicutes	ATCC 29212	-
Halomonas elongata	Gamma group	DSM 2581	-
Helicobacter pylori	Epsilon group	DSM 4867	-
Listeria monocytogenes	Firmicutes	DSM 20600	-
Micrococcus luteus	Firmicutes	DSM 1605	-

Pseudomonas aeruginosa	Gamma group	DSM 3227	-
Staphylococcus aureus	Firmicutes	ATCC 6538P	-
Staphylococcus epidermidis	Firmicutes	ATCC 12228	-
Vibrio proteolyticus	Gamma group	DSM 30189	-

Example 30
Sensitivity of the PCR quick test

In the experiments to determine the sensitivity of the PCR quick test for enterobacteriaceae, genomic *Escherichia coli* DNA of the ATCC 8739 strain was used representatively for the other enterobacteriaceae. According to these examinations, the detection width of the developed PCR quick test for enterobacteriaceae spans from less than 5 gfu (corresponding to 25 fg of genomic DNA) to more than 5,000,000 gfu (corresponding to 25 ng of genomic DNA) of *Escherichia coli* (Figure 10).

Even after 40 cycles, the no-template-controls (with no enterobacteriaceae DNA) gave no reaction with the PCR quick test developed herein.

Example 31
Product analysis

Sterile water for injection use (WFI, Lot No. 63022) was tested. The test result indicated absence of enterobacteriaceae DNA.

Example of 32
Failure variants in primer and probe sequences

Primer/probe combinations are defined as failure variants, which detect the target DNA sequences with non-satisfactory specificity (<100%) and sensitivity (<70%), such as the sequences specified in Example 27.

Literature relevant to the Examples:

Balows, A., Truper, H., Dworkin, M., Harder, W. & Schleifer, K.-H. (1991), The Prokaryotes: A Handbook on the Biology of Bacteria, Second Edition, Vol. 1-4, Springer Verlag, New York, NY.

Brosius, J., Palmer, J.L., Kennedy, J.P. & Noller, H.F. (1978), Complete Nucleotide Sequence of a 16S Ribosomal RNA Gene from *Escherichia coli*, Proc. Natl. Acad. Sci. USA 75, 4801-4805.

Holt, J. (editor in chief) (1989), Bergey's Manual of Systematic Bacteriology, First Edition, Vol. 1-4, Williams & Williams, Baltimore, MD.

Legends to the Figures

Legend to Fig. 1:
The DNA (10 ng per lane, 2-14) of all *S. aureus* strains employed (lanes 2-5) was detected by the *cap8-0* primers (# 15297 and # 15485). In contrast, the DNA of a closely related *Staphylococcus* species, i.e., *S. epidermidis* (lane 6) and that of other bacterial genera (lanes 7-11) was not detected. Fungus, fish and human DNAs (lanes 12-14) were used as controls, showing no detection signal. NTC (= no template control) is the water control wherein no DNA was used.

Legend to Fig. 6:
The DNA (1-10 ng) of all bacteria employed (*Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Salmonella typhimurium, Pseudomonas aeruginosa, and Streptococcus faecalis*) was detected by the 16S rRNA primer/probes set. When using genomic DNA (10 ng) of fungi (*Neurospora crassa*), plants (*Arabodopsis thaliana*) or humans (Perkin Elmer ABI 401846), the measured fluorescence radiation corresponded to that of the water control (no DNA control).

Legend to Fig. 7
Fluorescence radiation as a function of the amount of salmonella DNA employed. In the PCR quick test, salmonella DNA was used in amounts as isolated from 1-3, 50, 500 etc. germs. The amount of emitted fluorescence is given as so-called RQ value.
$RQ = (R^{+}/Q) - (R^{-}/Q)$

Legend to Fig. 8:
Water for injection use (10 ml analysis volume) was analyzed in four independent experiments for the presence of bacteria. 250 fg of genomic salmonella DNA (Fig. 8, farthest left) was used as positive control. In parallel, the

test product was spiked with 50 gfu/10 ml salmonella and then analyzed (each on the right). The individual results are illustrated.

Legend to Fig. 9

Schematic illustration of taxonomic relationships of enterobacteriaceae:

The individual genera of enterobacteriaceae belong to the gamma group of the proteobacteria which are classified as eubacteria. This scheme was the basis for the reflections relating to the specificity tests. To detect the specificity of the developed PCR quick test for enterobacteriaceae, members of the gamma group and some members of other groups of proteobacteria were predominantly used.

Legend to Fig. 10:

Sensitivity of the PCR quick test for enterobacteriaceae:

The obtained C_t values are illustrated as a function of germ-forming units (gfu) of enterobacteriaceae employed.

TaqMan

(1) GENERAL DECLARATIONS

(i) APPLICANT:

(A) NAME: **SCHERING AKTIENGESELLSCHAFT**

(B) STREET: **MÜLLERSTRASSE 178**

(C) CITY: **13353 BERLIN**

(E) COUNTRY: **GERMANY**

(F) POSTAL CODE: **D-13353**

(ii) Title of invention: A method of detecting microorganisms in products

(iii) NUMBER of SEQUENCES: 54

(iv) COMPUTER READABLE FORM

(A) MEDIUM TYPE: **FLOPPY DISK**

(B) COMPUTER: **486/INTEL**

(C) OPERATING SYSTEM: **WINDOWS**

(D) SOFTWARE: **WINWORD;**

(v) CURRENT APPLICATION INFORMATION:

For all SEQ ID NO 1 to 54:

TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
HYPOTHETICAL: no
ANTISENSE: no

(2) SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS

LENGTH: 214 Nucleotides
MOLEKULE TYPE: Primer-Sonde-Primer
SOURCE ORGANISM NAME: Staphylococcus aureus
FEATURE: Primer-Sonde-Primer for Staphylococcus aureus

SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
AGATGCACGT ACTGCTGAAA TGAGTAAGCT AATGGAAAAC      040
ACATATAGAG ACGTGAATAT TGCTTTAGCT AATGAATTAA      080
CAAAAATTTG CAATAACTTA AATATTAATG TATTAGTTGT      120
GATTGAAATG GCAAACAAAC ATCCGCGTGT TAATATCCAT      160
CAACCTGGTC CAGGAGTAGG CGGTCATTGT TTAGCTGTTG      200
ATCCGTACTT TATT                                 214
```

(2) SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS

LENGTH: 310 Nucleotides
MOLEKULE TYPE: Primer-Sonde-Primer

TaqMan

SOURCE ORGANISM NAME: Staphylococcus aureus
FEATURE: Primer-Sonde-Primer for Pseudomonas aeruginosa

SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
CAGGCCTTCG ATGCCCTGAG CGGTATTCAG GCACCGGCGC      040
CCAACGCCGA AGAACTCCAG CATTTCTGCC AATTGCTGCT      080
GGACTATGTA TCTGCCGGAC ACTTCGAGGT CTACGAGCAA      120
CTGACGGCGG AAGGCAAGGC CTTCGGCGAT CAGCGCGGCC      160
TGGAGCTGGC CAAGCAGATC TTCCCCCGGC TGGAAGCCAT      200
CACCGAATCC GCGCTGAACT TCAACGACCG CTGCGACAAC      240
GGCGATTGCC GTGAAGGAGC CTGCCTCATC GCGGAGCTGA      280
AGGTCCTGCG GCAACAGTTG CACGAACGCT                310
```

(2) SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS

LENGTH: 222 Nucleotides
MOLEKULE TYPE: Primer-Sonde-Primer
SOURCE ORGANISM NAME: Staphylococcus aureus
FEATURE: Primer-Sonde-Primer for Escherichia coli

SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
AAAGTAGAAC GTAATGGTTC TGTGCATATT GATGCCCGCG      040
ACGTTAATGT ATTCTGCGCA CCTTACGATC TGGTTAAAAC      080
CATGCGTGCT TCTATCTGGG CGCTGGGGCC GCTGGTAGCG      120
CGCTTTGGTC AGGGGCAAGT TTCACTACCT GGCGGTTGTA      160
CGATCGGTGC GCGTCCGGTT GATCTACACA TTTCTGGCCT      200
CGAACAATTA GGCGCGACCA TC                        222
```

(2) SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS

LENGTH: 310 Nucleotides
MOLEKULE TYPE: Primer-Sonde-Primer
SOURCE ORGANISM NAME: Salmonella ssp.
FEATURE: Primer-Sonde-Primer for Salmonella ssp.

SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
TGATTGAAGC CGATGCCGGT GAAATTATCG CCACGTTCGG  040
GCAATTCGTT ATTGGCGATA GCCTGGCGGT GGGTTTTGTT  080
GTCTTCTCTA TTGTCACCGT GGTCCAGTTT ATCGTTATTA  120
CCAAAGGTTC AGAACGTGTC GCGGAAGTCG CGGCCCGATT  160
TTCTCTGGAT GGTATGCCCG GTAAACAGAT GAGTATTGAT  200
GCCGATTTGA AGGCCGGTAT TATTGATGCG GATGCCGCGC  240
GCGAACGGCG AAGCGTACTG GAAAGGGAAA GCCAGCTTTA  280
CGGTTCCTTT GACGGTGCGA TGAAGTTTAT             310
```

(2) SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS

LENGTH: 356 Nucleotides
MOLECULE TYPE: Primer-Sonde-Primer
SOURCE ORGANISM NAME: Bacteria
FEATURE: Primer-Sonde-Primer for Bacteria

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SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCATGGCTGT CGTCAGCTCG TGTTGTGAAA TGTTGGGTTA 040
AGTCCCGCAA CGAGCGCAAC CCTTATCCTT TGTTGCCAGC 080
GGTCCGGCCG GGAACTCAAA GGAGACTGCC AGTGATAAAC 120
TGGAGGAAGG TGGGGATGAC GTCAAGTCAT CATGGCCCTT 160
ACGACCAGGG CTACACACGT GCTACAATGG CGCATACAAA 200
GAGAAGCGAC CTCGCGAGAG CAAGCGGACC TCATAAAGTG 240
CGTCGTAGTC CGGATTGGAG TCTGCAACTC GACTCCATGA 280
AGTCGGAATC GCTAGTAATC GTGGATCAGA ATGCCACGGT 320
GAATACGTTC CCGGGCCTTG TACACACCGC CCGTCA 356

(2) SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS

LENGTH: 24 Nucleotides
MOLECULE TYPE: Primer cap-8 forward # 15297*)
FEATURE: Primer cap-8 forward # 15297*)

SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AGATGCACGT ACTGCTGAAA TGAG 024

(2) SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS

LENGTH: 20 Nucleotides
TYPE: Nucleotid sequence
STRANDEDNESS: single
TOPOLOGY: linear
HYPOTHETICAL: no
ANTISENSE: no
MOLECULE TYPE: Sonde cap-8 # 15460*
FEATURE: Sonde cap-8 # 15460*, used as reverse complement, TAMRA before and FAM after the Sequence

SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CCTGGTCCAG GAGTAGGCGG 020

(2) SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS

LENGTH: 26 Nucleotides
MOLECULE TYPE: Primer cap-8 reverse # 15485
FEATURE: Primer cap-8 reverse # 15485* used as reverse complement

SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GTTTAGCTGT TGATCCGTAC TTTATT 026

(2) SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS

LENGTH: 23 Nucleotides
MOLECULE TYPE: Primer *algQ* forward # 876*
FEATURE: Primer *algQ* forward # 876*

SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CTTCGATGCC CTGAGCGGTA TTC 023

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(2) SEQ ID NO: 10:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 26 Nucleotides
MOLECULE TYPE: Sonde *algQ* # 911
FEATURE: Sonde *algQ* # 911, FAM before and TAMRA after the Sequence
SEQUENCE DESCRIPTION: SEQ ID NO: 10:
CCAACGCCGA AGAACTCCAG CATTTC **026**

(2) SEQ ID NO: 11:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 23 Nucleotides
MOLECULE TYPE: Reverse Primer Sequence (# 1147):
FEATURE: Primer *algQ* reverse # 1147* used as reverse complement
SEQUENCE DESCRIPTION: SEQ ID NO: 11:
CTGAAGGTCC TGCGGCAACA GTT **023**

(2) SEQ ID NO: 12:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 24 Nucleotides
MOLECULE TYPE: Forward Primer Sequence (# 767*):
FEATURE: Forward Primer Sequence (# 767*):
SEQUENCE DESCRIPTION: SEQ ID NO: 12:
GTTCTGTGCA TATTGATGCC CGCG **024**

(2) SEQ ID NO: 13:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 23 Nucleotides
MOLECULE TYPE: Sonde (# 802)
FEATURE: Sonde (# 802), FAM before and RAMARA after the Sequence
SEQUENCE DESCRIPTION: SEQ ID NO: 13:
TCTGCGCACC TTACGATCTG GTT **023**

(2) SEQ ID NO: 14:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 24 Nucleotides
MOLECULE TYPE: Reverse Primer Sequence (# 884)
FEATURE: Reverse Primer Sequence (# 884) used as reverse complement
SEQUENCE DESCRIPTION: SEQ ID NO: 14:
GCAAGTTTCA CTACCTGGCG GTTG **024**

(2) SEQ ID NO: 15:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 24 Nucleotides
MOLECULE TYPE: Forward Primer Sequence (# 269*)
FEATURE: Forward Primer Sequence (# 269*)
SEQUENCE DESCRIPTION: SEQ ID NO: 15:
GTGAAATTAT CGCCACGTTC GGGC **024**

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(2) SEQ ID NO: 16:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 24 Nucleotides
MOLECULE TYPE: Sonde (# 333)
FEATURE: Sonde (# 333), FAM before and TAMARA after the Sequence
SEQUENCE DESCRIPTION: SEQ ID NO: 16:
CTTCTCTATT GTCACCGTGG TCCA **024**

(2) SEQ ID NO: 17:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 24 Nucleotides
MOLECULE TYPE: Reverse Primer Sequence (# 542)
FEATURE: Reverse Primer Sequence (# 542) used as reverse complement
SEQUENCE DESCRIPTION: SEQ ID NO: 17:
GGTTCCTTTG ACGGTGCGAT GAAG **024**

(2) SEQ ID NO: 18:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 19 Nucleotides
MOLECULE TYPE: Primer 16S rRNA forward # 1053*
FEATURE: Primer 16S rRNA forward # 1053*
SEQUENCE DESCRIPTION: SEQ ID NO: 18:
GCATGGCTGT CGTCAGCTC **019**

(2) SEQ ID NO: 19:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 23 Nucleotides
MOLECULE TYPE: Sonde 16S rRNA # 1090
FEATURE: Sonde 16S rRNA # 1090, FAM before and TAMARA after the Sequence
SEQUENCE DESCRIPTION: SEQ ID NO: 19:
TTAAGTCCCG CAACGAGCGC AAC **023**

(2) SEQ ID NO: 20:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 20 Nucleotides
MOLECULE TYPE: Primer 16S rRNA reverse # 1386*
FEATURE: Primer 16S rRNA reverse # 1386*
SEQUENCE DESCRIPTION: SEQ ID NO: 20:
TGACGGGCGG TGTGTACAAG **020**

(2) SEQ ID NO: 21:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 23 Nucleotides
MOLECULE TYPE: Sonde
FEATURE: Sonde of Salmonella ssp
SEQUENCE DESCRIPTION: SEQ ID NO: 21:
TTTGTTATTG GCGATAGCCT GGC **023**

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(2) SEQ ID NO: 22:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 23 Nucleotides
MOLECULE TYPE: Sonde
FEATURE: Sonde of Salmonella ssp.
SEQUENCE DESCRIPTION: SEQ ID NO: 22:
TTCTCTGGAT GGTATGCCCG GTA **023**

(2) SEQ ID NO: 23:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 25 Nucleotides
MOLECULE TYPE: Reverse Primer
FEATURE: Reverse Primer for Staphylococcus aureus
SEQUENCE DESCRIPTION: SEQ ID NO: 23:
CATTGTTTAG CTGT TGATCC GTAC T **025**

(2) SEQ ID NO: 24:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 24 Nucleotides
MOLECULE TYPE: Primer
FEATURE: Forward Primer *for Staphylococcus aureus*
SEQUENCE DESCRIPTION: SEQ ID NO: 24:
GCACGT ACTG CTGAAA TGAG TAAG **024**

(2) SEQ ID NO: 25:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 21 Nucleotides
MOLECULE TYPE: Primer
FEATURE: Forward Primer for Pseudomonas aeruginosa
SEQUENCE DESCRIPTION: SEQ ID NO: 25:
CAGGCCTTCG ATGCCCTGAG C **021**

(2) SEQ ID NO: 26:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 22 Nucleotides
MOLECULE TYPE: Primer
FEATURE: Reverse Primer *for Pseudomonas aeruginosa*
SEQUENCE DESCRIPTION: SEQ ID NO: 26:
GCTGAAGGTC CTGCGGCAAC AG **022**

(2) SEQ ID NO: 27:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 23 Nucleotides
MOLECULE TYPE: Primer
FEATURE: Forward Primer for E. coli
SEQUENCE DESCRIPTION: SEQ ID NO: 27:
TAGAACGTAA TGGTTCTGTG CAT **023**

(2) SEQ ID NO: 28:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 23 Nucleotides
MOLECULE TYPE: Primer

TaqMan

FEATURE: Reverse Primer for *E. coli*
SEQUENCE DESCRIPTION: SEQ ID NO: 28:
CTGGCCTCGA ACAATTAGGC GCG **023**

(2) SEQ ID NO: 29:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 23 Nucleotides
MOLECULE TYPE: Primer
FEATURE: Forward Primer for Bacteria
SEQUENCE DESCRIPTION: SEQ ID NO: 29:
TGCATGGCTG TCGTCAGCTC **020**

(2) SEQ ID NO: 30:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 23 Nucleotides
MOLECULE TYPE: Sonde
FEATURE: Sonde for Bacteria in general
SEQUENCE DESCRIPTION: SEQ ID NO: 30:
TTGGGTTAAG TCCCG CAACG AGC **033**

(2) SEQ ID NO: 31:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 22 Nucleotides
MOLECULE TYPE: Primer
FEATURE: Forward Primer for *Staphylococcus aureus*
SEQUENCE DESCRIPTION: SEQ ID NO: 31:
ATGCACGTAC TGCTGAAATG AG **032**

(2) SEQ ID NO: 32:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 26 Nucleotides
MOLECULE TYPE: Sonde
FEATURE: Sonde for *Staphylococcus aureus*
SEQUENCE DESCRIPTION: SEQ ID NO: 32:
AACACATATA GAGACGTGAA TATTGC **035**

(2) SEQ ID NO: 33:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 22 Nucleotides
MOLECULE TYPE: Primer
FEATURE: Reverse Primer for *Staphylococcus aureus*
SEQUENCE DESCRIPTION: SEQ ID NO: 33:
GTTTAGCTGT TGATCCGTAC TT **022**

(2) SEQ ID NO: 34:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 25 Nucleotides
MOLECULE TYPE: Sonde
FEATURE: Sonde for *Pseudomonas aeruginosa*
SEQUENCE DESCRIPTION: SEQ ID NO: 34:
CAATTGCTGC TGGACTATGT ATCTG **025**

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(2) SEQ ID NO: 35:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 25 Nucleotides
MOLECULE TYPE: Primer
FEATURE: Forward Primer for *Pseudomonas aeruginosa*
SEQUENCE DESCRIPTION: SEQ ID NO: 35:
CAACGCCGAA GAACTCCAGC ATTTC **025**

(2) SEQ ID NO: 36:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 27 Nucleotides
MOLECULE TYPE: Sonde
FEATURE: Sonde for *Pseudomonas aeruginosa*
SEQUENCE DESCRIPTION: SEQ ID NO: 36:
AACGCCGA AG AACTCCAG CA TTTCTGC **027**

(2) SEQ ID NO: 37:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 22 Nucleotides
MOLECULE TYPE: Primer
FEATURE: Reverse Primer for *Escherichia coli*
SEQUENCE DESCRIPTION: SEQ ID NO: 37:
CATTTCTGGC CTCGAACAAT TA **022**

(2) SEQ ID NO: 38:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 23 Nucleotides
MOLECULE TYPE: Sonde
FEATURE: Sonde for *Escherichia coli*
SEQUENCE DESCRIPTION: SEQ ID NO: 38:
CCGCTGGTAG CGCGTTTTGG TCA **023**

(2) SEQ ID NO: 39:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 23 Nucleotides
MOLECULE TYPE: Primer
FEATURE: Forward Primer *for Escherichia coli*
SEQUENCE DESCRIPTION: SEQ ID NO: 39:
ATGAAGCTGC TAAGCCAGCT GGG **023**

(2) SEQ ID NO: 40:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 25 Nucleotides
MOLECULE TYPE: Primer
FEATURE: Forward Primer for *Salmonella ssp*
SEQUENCE DESCRIPTION: SEQ ID NO: 40:
TTGAAGCCGA TGCCGGTGAA ATTAT **025**

(2) SEQ ID NO: 41:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 24 Nucleotides
MOLECULE TYPE: Sonde

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FEATURE: Sonde for *Salmonella ssp*
SEQUENCE DESCRIPTION: SEQ ID NO: 41:
TTTGTTGTCT TCTCTATTGT CACC **024**

(2) SEQ ID NO: 42:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 20 Nucleotides
MOLECULE TYPE: Primer
FEATURE: Reverse Primer for Bacteria in general
SEQUENCE DESCRIPTION: SEQ ID NO: 42:
AAGTCGTAAC AAGGTAACCA **020**

(2) SEQ ID NO: 43:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 21 Nucleotides
MOLECULE TYPE: Primer
FEATURE: Forward Primer for Bacteria in general
SEQUENCE DESCRIPTION: SEQ ID NO: 43:
GGATTAGATA CCCTGGTAGT C **021**

(2) SEQ ID NO: 44:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 20 Nucleotides
MOLECULE TYPE: Forward-Primer (#1053)
SOURCE ORGANISM NAME: Enterobacteriaceae
FEATURE: Forward-Primer (#1053) for Enterobacteriaceae
SEQUENCE DESCRIPTION: SEQ ID NO: 44:
GCATGGCTGT CGTCAGCTC **20**

(2) SEQ ID NO: 45:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 20 Nucleotides
MOLECULE TYPE: Reverse-Primer (#1270)
SOURCE ORGANISM NAME: Enterobacteriaceae
FEATURE: Reverse-Primer (#1270) for Enterobacteriaceae
SEQUENCE DESCRIPTION: SEQ ID NO: 45:
TTTATGAGGT CCGCTTGCTC **45**

(2) SEQ ID NO: 46:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 23 Nucleotides
MOLECULE TYPE: Sonde (#1090)
SOURCE ORGANISM NAME: Enterobacteriaceae
FEATURE: Sonde (#1090) for Enterobacteriaceae
SEQUENCE DESCRIPTION: SEQ ID NO: 46:
TTAAGTCCCG CAACGAGCGC AAC **23**

(2) SEQ ID NO: 47:
(i) SEQUENCE CHARACTERISTICS
LENGTH: 19 Nucleotides
MOLECULE TYPE: (Forward-Primer #1053)

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SOURCE ORGANISM NAME: Targetsequence of 16S rRNA Gene of Enterobacteriaceae

FEATURE: (Forward-Primer #1053) for Enterobacteriaceae

SEQUENCE DESCRIPTION: SEQ ID NO: 47:

GCATGGCTGT CGTCAGCTC **19**

(2) SEQ ID NO: 48:

(i) SEQUENCE CHARACTERISTICS

LENGTH: 23 Nucleotides

MOLECULE TYPE: (Sonde #1090)

SOURCE ORGANISM NAME: Enterobacteriaceae

FEATURE: (Sonde #1090) for Enterobactereaceae

SEQUENCE DESCRIPTION: SEQ ID NO: 48:

TTAAGTCCCG CAACGAGCGC AAC **23**

(2) SEQ ID NO: 49:

(i) SEQUENCE CHARACTERISTICS

LENGTH: 19 Nucleotides

MOLECULE TYPE: (Reverse-Primer #1270)

SOURCE ORGANISM NAME: Enterobacteriaceae

FEATURE: (Reverse-Primer #1270) for Enterobacteriaceae

SEQUENCE DESCRIPTION: SEQ ID NO: 49:

TCGTTCGCCT GGAGTATTT **19**

(2) SEQ ID NO: 50:

(i) SEQUENCE CHARACTERISTICS

LENGTH: 20 Nucleotides

MOLECULE TYPE: Forward-Primer

SOURCE ORGANISM NAME: Enterobacteriaceae

FEATURE: Forward-Primer for Enterobacteriaceae as failure sequence

SEQUENCE DESCRIPTION: SEQ ID NO: 50:

GTGCTGCATG GCTGTCGTC **20**

(2) SEQ ID NO: 51:

(i) SEQUENCE CHARACTERISTICS

LENGTH: 23 Nucleotides

MOLECULE TYPE: Sonde

SOURCE ORGANISM NAME: Enterobacteriaceae

FEATURE: Sonde for Enterobacteriaceae as failure sequence

SEQUENCE DESCRIPTION: SEQ ID NO: 51:

AGTCCCGCAA CGAGCGCAAC CC **23**

(2) SEQ ID NO: 52:

(i) SEQUENCE CHARACTERISTICS

LENGTH: 23 Nucleotides

MOLECULE TYPE: Sonde

SOURCE ORGANISM NAME: Enterobacteriaceae

FEATURE: Sonde for Enterobacteriaceae as failure sequence

SEQUENCE DESCRIPTION: SEQ ID NO: 52:

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ATGTTGGGTT AAGTCCCGCA ACG **23**

(2) SEQ ID NO: 53:

(i) SEQUENCE CHARACTERISTICS

LENGTH: 20 Nucleotides
MOLECULE TYPE: Forward-Primer
SOURCE ORGANISM NAME: Enterobacteriaceae
FEATURE: Forward-Primer for Enterobacteriaceae as failure sequence

SEQUENCE DESCRIPTION: SEQ ID NO: 53:

GCTGTCGTCA GCTCGTGTT **20**

(2) SEQ ID NO: 54:

(i) SEQUENCE CHARACTERISTICS

LENGTH: 21 Nucleotides
MOLECULE TYPE: Reverse-Primer
SOURCE ORGANISM NAME: Enterobacteriaceae
FEATURE: Reverse-Primer for Enterobacteriaceae as failure sequence

SEQUENCE DESCRIPTION: SEQ ID NO: 54:

AACTTTATGA GGTCCGCTTG C **21**